IDENTIFICATION OF NOVEL FACTORS INVOLVED IN MES-POLYCOMB MEDIATED TRANSCRIPTIONAL REPRESSION

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Development of multicellular organisms requires precise coordination of gene expression, regulated by alterations in genomic accessibility and chromatin structure. Changes in chromatin architecture also mediate the transition from plasticity to cell fate commitment. This work describes a candidate RNAi screen in *C.elegans* designed to identify factors functioning in transcriptional silencing, based on enhancement/suppression of phenotypes associated with mutants in three major transcriptional silencing pathways: *mes-3*/Polycomb, *met-2*/SET-DB1, and *hpl-1; hpl-2*/HP1.

Strikingly, all known members of the Mes/Polycomb pathway of transcriptional repression, including members of PRC2 (*mes-2/mes-3/mes-6*), *mes-4,* and *set-2* strongly suppress the *hpl-1; hpl-2* larval arrest phenotype. A number of additional factors also strongly suppressed *hpl-1; hpl-2* arrest, including members of the COMPASS/MLL complex (H3K4me), members of the NuA4 HAT complex, members of SWR1 nucleosome remodeling complex (incorporation of H2A.Z), O-GlcNAc transferase (catalyzes a type of posttranslational modification), a novel factor (T19B4.5), and two proteins involved in DNA repair.

Based on the hypothesis that the strong *hpl-1; hpl-2* suppression signature indicates association with the Mes pathway, these genes were tested in a number of secondary analyses related to Mes function, including maternal effect sterility, germline

morphology, H3K27 methylation, interaction in the SynMuv pathway of vulval specification, suppression of *mep-1*/NuRD larval lethality, and desilencing of tandem arrays in the germline. Phenotypes associated with these assays suggest that *hpl-1; hpl-2* suppressors can be divided into two classes based on similarity to either MES-4 or PRC2 phenotypes.

Data suggest that the activating complex(es) NuA4/SWR1 interact with MES-4, and may contribute to repression of X linked gene expression. Our analyses also indicate that the COMPASS complex likely functions in the Mes/PcG pathway in two or more forms, one of which is independent of SET-2. Phenotypes associated with SET-2-independent COMPASS complex correlate well with *mes-4*, while SET-2 may interact more closely with MES/PRC2.

This work also demonstrates a novel phenotype associated with the coiled-coil protein T19B4.5. Loss of this factor induces upregulation of let-858::GFP transgenic constructs in the soma, coupled with reduced expression in the germ line. These data suggest that T19B4.5 may modulate both the SynMuv and Mes pathways in a context dependent manner.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Overview

Gene expression in eukaryotes is regulated in part by alteration of genomic accessibility and chromatin structure. Chromatin is made up of DNA that is wrapped around a histone octamer core, organizing the DNA into less compact transcriptionally active and more compact quiescent domains. Each histone has a labile tail that is subject to posttranslational modifications which function to regulate chromatin compaction and transcriptional output. Histone modifications can also act as binding platforms for chromatin remodeling complexes that induce further changes in chromatin structure.

The Polycomb Repressive Complex PRC2 mediates trimethylation of histone 3 lysine 27 (H3K27me3), a mark that is strongly correlated with transcriptional silencing and chromatin compaction (Campos & Reinburg, 2009; Simon & Kingston, 2009; Yuzyuk et al., 2006). However, the mechanisms regulating transcriptional repression downstream of the H3K27me3 mark remain unclear. Although controversial, a complex known as PRC1 is thought to bind H3K27me3 and induce PRC2 mediated transcriptional silencing by ubiquitination and nucleosome compaction (Margueron and Reinberg, 2011). Yet some species, including *C.*

elegans and plants, appear to lack a functional PRC1 complex (Wenzel et al., 2011; Simon and Kinston, 2009). Moreover, PRC1 is not present at all PRC2 silenced loci in flies and mammals (Ku et al., 2008; Schwartz et al., 2006; Boyer et al., 2006; Leeb et al 2010, Schoeftner et al., 2006). These data suggest that additional factors cooperate in PRC2 induced transcriptional repression.

The *C.elegans* PRC2 complex is comprised of MES-2/E(z), MES-6/Esc, and the novel factor MES-3 (Bender et al., 2004). As in other species, the *C. elegans* PRC2 complex mediates H3K27 trimethylation, and is required for H3K27me3 marks in much of the germ line and during early embryogenesis (Bender et al., 2004). In addition to maternal effect sterility (*mes*) induced by germ line necrosis and loss of H3K27me3, *mes* deficiency results in several phenotypes, including derepression of high copy transgenes in the germline, changes in RNAi efficacy, and suppression of the synthetic multivulva (SynMuv) phenotype (Capowski et al., 1991, Kelly et al., 1998, Wang et al., 2005; Kim et al., 2005, Cui et al., 2006). Previous studies have suggested that the phenotypes associated with loss of PRC2 complex function result from derepression of X-linked genes, as H3K27me3 is enriched on the X chromosome in the wildtype *C. elegans* germ line (Bender, 2004). Further, in the absence of PRC2 complex function, activating marks become ectopically localized to the X chromosomes (Fong et al., 2002).

Silencing of the X chromosomes in the adult germ line also requires the conserved methlytransferase MES-4, which catalyzes methylation of histone 3 on lysine 36 (H3K367me2/3), a mark generally associated with transcriptional activation (Bender et al., 2006). Loss of MES-4 phenocopies loss of PRC2

components, and functional PRC2 is required to restrict MES-4 activity to autosomes (Bender et al., 2006; Fong et al., 2006). It is currently unclear how MES-4 and PRC2, which have seemingly opposing functions, repress X-linked transcriptional activity and regulate chromatin dynamics in the germ line and early embryo. In addition, and in contrast to the germ line, loss of Polycomb-mediated transcriptional silencing has very subtle effects in somatic tissues. This is surprising, given the global role of Polycomb repressor complexes in other organisms. Together, these data suggest that additional silencing factors might be required to regulate transcriptional silencing in *C. elegans*.

SynMuv factors represent one interesting class of candidates that may function coincident to the MES pathway of transcriptional silencing. SynMuv factors represent a large group of proteins, many of which are involved in chromatin structure and regulation of gene expression. A number of these factors are associated with loss of function phenotypes similar to those of *mes* mutants, including changes in RNAi efficacy and germ line expression of transgenes. Loss of the SynMuv factor HP1 (heterochromatin protein 1), encoded by the partially redundant *hpl-1* and *hpl-2,* induces temperature sensitive larval arrest, a phenotype that is suppressed by loss of *mes-2*, *mes-3*, *mes-6*, or *mes-4* (Schott et al., 2006; Simonet et al., 2007). This genetic interaction suggests that the PRC2/Mes pathway may function in conjunction with HP1 to regulate patterns of gene expression during development and germ line maturation.

To identify additional candidates functioning in transcriptional silencing in *C. elegans*, we performed a candidate RNAi screen and isolated genetic enhancers of

mes-3/Polycomb as well as enhancers and suppressors of *hpl-1; hpl-2*/HP1. Two classes of genes were predicted to function in PRC2-mediated transcriptional repression: 1) genes that enhance *mes-3* maternal effect sterility (similar to *set-2)* (Xu and Strome, 2001); and 2) genes that suppress the *hpl-1; hpl-2* temperature sensitive larval arrest phenotype (similar to loss of the Polycomb genes). Of the 738 RNAi clones tested, loss of 97 genes resulted in enhancement of the *mes-3* phenotype, while only 17 resulted in strong suppression of *hpl-1; hpl-2*. The *hpl-1; hpl-2* suppressor class includes all factors previously shown to function in the MES/PRC2 pathway, demonstrating the strength of our approach. Intriguingly, the *hpl-1; hpl-2* suppressor category also contains multiple components of two complexes generally associated with transcriptional activation: the COMPASS/MLL complex, which functions as a histone 3 lysine 4 (H3K4) methylation and regulatory complex, and the NuA4/SWR1 complex, which mediates the acetylation of histone 4 as well as incorporation of the histone variant H2A.Z.

We further characterized the *hpl-1; hpl-2* suppression cohort in order to define the role of these activation complexes within the context of MES/PRC2 mediated transcriptional silencing. These secondary analyses included testing for changes in localization and concentration of chromatin marks, germ line expression of a transgenic array, and a number of additional assays for *mes*-related phenotypes. We show that phenotypes associated with loss of COMPASS/MLL and NuA4/SWR1 complex function appear closely correlated to loss of MES-4. Based on our findings, we hypothesize that the primary role of the PRC2 complex is not simply to inhibit X-

linked gene expression, but also to aid in targeting activation machinery such as COMPASS/MLL and NuA4/SWR1 to specific loci.

Chromatin dynamics, transcriptional regulation, and cellular plasticity

Chromatin and transcriptional regulation

Gene expression in eukaryotes is regulated in part by alteration of genomic accessibility and chromatin structure, which are controlled by a number of mechanisms. Chromatin compaction is highly dynamic; euchromatic DNA is accessible to interacting factors and associated with active transcription, while heterochromatic domains are generally silent. Eukaryotic chromatin organization is mediated by the formation of nucleosomes, 146 base pairs of DNA wrapped around a histone octamer core composed of two copies each of H2A, H2B, H3 and H4. Histone H1 functions as a linker histone, and aids the compaction of nucleosomes thought to limit accessibility to transcriptional regulation, replication, and repair machinery.

The reversible posttranslational modification (PTM) of histone tails is thought to be one of the driving forces behind changes in genomic structure. Histone tail modifications include phosphorylation, methylation, acetylation, ubiquitination, sumoylation, and poly-ADP ribosylation. These modifications are tightly regulated by a number of enzymes including kinases, methyltransferases (HMTs), demethylases, histone acetyltransferases (HATs), deacetylases (HDACs), ubiquitin ligases, and others (Kouzarides, 2007). The type of mark, the residue modified, and the level of modification (i.e., mono, di, trimethylation) are key determinants of the resulting transcriptional readout (Kouzarides, 2007). In addition to the introduction of direct steric

changes, PTMs also serve as docking sites for "reader" proteins and complexes. Different modifications can induce or inhibit subsequent PTMs and effector interactions; this cross- talk occurs both in *cis* and in *trans* (Lee et al., 2010). The modification of histone tails and the association of effector molecules can influence local chromatin architecture, as well as promote or inhibit transcriptional activity; however, much of the data is strictly correlative.

In addition to the somewhat controversial "histone code" of post-translation modifications, chromatin dynamics are also controlled by nucleosome remodeling complexes and the incorporation of histone variants (i.e., H2A.Z, H3.3) which promote nucleosome diversity and influence chromatin structure by altering interactions between DNA and nucleosomes and/or changing the accessibility of genomic elements to regulatory factors. Nucleosome remodelers are enzyme-containing complexes that utilize ATP hydrolysis to facilitate histone octamer sliding, removal, and/or exchange of histone variants. Chromatin remodeling complexes are divided into several subclasses based on the structure of the ATPase domain; these include the SWI/SNF, IWSI, CHD, and INO80 families (Lu et al., 2009). In yeast, the INO80 family contains the NuA4 complex and SWR1, but in mammals and *Drosophila*, these function as a single complex called Tip60-p400, capable of both remodeling and HAT activity (Hargreaves & Crabtree, 2011).

Types of chromatin

Active chromatin, or euchromatin, is associated with productive transcription and correlates with engaged RNA Polymerase II (PolII) and a specific cohort of histone

marks. Generally, the promoter regions of actively transcribed genes contain H3K4me3, a mark catalyzed by Trithorax group proteins. Elongation of RNA PolII is correlated with the deposition of H3K36me2/3 across gene bodies, while H3K4me3 and H3K36me2/3 correlate with the initiation and elongation phases of transcription (Schaner & Kelly, 2006). Acetylation of histone tails is also associated with gene activity, and has been shown to sterically alter the nucleosome-DNA interaction, resulting in an open configuration thought to allow processivity of the RNA PolII complex (Campos & Reinburg, 2009). Together, these marks define transcribed promoters and block off-target interactions between polymerase and gene bodies.

As shown in Figure 1, heterochromatin is defined by condensation of nucleosome bound DNA and associated proteins, forming a structure that is relatively stable and refractory to transcriptional machinery. Heterochromatin is found in at least two forms. The first is constitutive heterochromatin, which is gene-poor, globally silenced throughout development, and enriched with repetitive DNA sequences and transposable

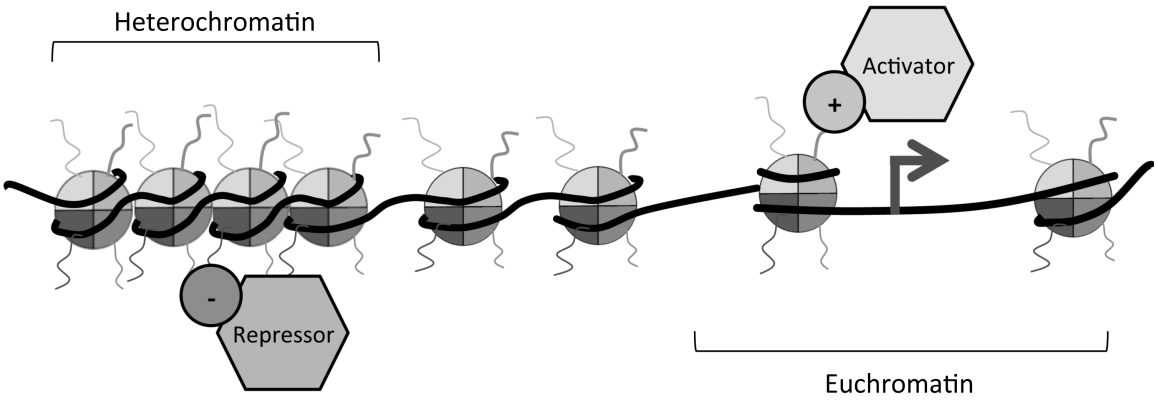


Figure 1. Different forms of chromatin. Nucleosomes in heterochromatin are highly compact, and histone tails are modified with repressive marks and associated with co-factors supporting the condensed state. Euchromatic DNA is open and associated with transcriptional activity.

elements, suggesting that this type of domain evolved as a mechanism of genomic stability. Constitutive heterochromatin is capable of silencing nearby genes (i.e., position-effect variegation), but appears to function primarily in the organization of nuclear sub-domains and chromosomal structures including telomeres and centromeres (Grewal & Moazed 2003).

Facultative heterochromatin includes regions of the genome targeted for silencing in a context-specific manner, including individually silenced loci that are repressed in a spatial and/or temporal-specific manner. Genes silenced by facultative heterochromatin can be expressed in response to specific cues, and regulation of gene expression by heterochromatin may play an important role in cell fate specification and differentiation (Jenuwein & Allis, 2001).

Some histone modification states are shared by both constitutive and facultative heterochromatin, including hypoacetylation and H3K9me2/3. However, H3K9me3 (trimethyl) is generally associated with constitutive heterochromatin, while H3K9me2 (dimethyl) correlates with facultative silencing in most organisms (Trojer & Reinberg, 2007). Similarly, H4K20me3 and H3K27me1 mark constitutive heterochromatin, but H4K20me1 and H3K27me2/3 are associated primarily with facultative repression (Campos & Reinburg, 2009). Heterochromatin protein 1 (HP1) has classically been associated with stabilization of constitutive silencing, but the role of HP1 has become somewhat less clear as different isoforms have been shown to function in a number of processes, including DNA repair and even active transcription (Kwon & Workman, 2011). Therefore, while both types of heterochromatin are involved in repression, they

are likely associated with different effector molecules and carry out different functions within a cell.

Heterochromatin is an important feature of the genomic landscape, yet the mechanisms directing heterochromatin formation are currently unclear, as are the mechanisms responsible for heterochromatin remodeling and gene expression. Current hypotheses suggest that directed heterochromatin formation may function during cell fate commitment and lineage specification.

Transcriptional silencing: Plasticity to commitment

One of the central questions facing developmental biologists today concerns the mechanisms mediating developmental potential and its loss during the onset of differentiation. Pluripotency refers to the ability of a cell to differentiate into any of the three germ layers, and is often associated with the ability to self-renew, as has been observed for ES cells derived from a transient population of pluripotent cells in the blastocyst and cultured *in vitro*. Within the context of an organism, pluripotent cells must proliferate, commit to specific cell fates, and become progressively restricted in developmental potential in order to generate a fully developed animal. In addition, a subset of pluripotent cells must be compartmentalized (or be regenerated in some systems), in order to retain pluripotency and reproductive potential. As cells become developmentally restricted and begin to acquire positional and cell type identity, global changes in chromatin structure occur concomitantly with changes in gene expression. Loss of pluripotency has been correlated with decreased transcriptional permissivity,

increased heterochromatic foci by EM, and the establishment of heritable gene expression programs (Mattout & Meshor; Efroni et al., 2008).

The pathways controlling cell fate specification are well studied, as are the transcription factors involed in induction and maintenance of pluripotency. However, the mechanisms coupling loss of pluripotency with cell fate establishment remain largely unexplored. While ES cells represent an important model with which to study pluripotency, it is also important to address the mechanisms governing the transition from plasticity to cell fate specification in *in vivo* models such as *C.elegans*. This system allows for a better understanding of endogenous mechansims, taking into account developmental timing, cell to cell interactions, etc., in cells that are not articially induced or maintained.

Reorganization of chromatin during cell fate commitment

One striking difference between pluripotent cells and differentiated cells is the makeup of the nuclear architecture. By electron microscopy, the nucleoplasm of pluripotent cells appears diffuse; this open configuration is associated with euchromatin (Niwa, 2007; Leung et al., 1999). The chromatin of embryonic stem (ES) cells is highly sensitive to nuclease activity, indicative of weak interactions between DNA, histone octamers, and structural proteins (Gaspar-Maia et al., 2011). Chromatin in ES cells is also enriched with histone modifications associated with active transcription (including H3K4me3, H3K9ac3, and H4Ac). For instance, H3K4 trimethylation is detected at 80% of annotated promoters in ES cells, while RNA PolII marks more than 50% of promoters, including transcriptionally silent loci (Guenther et al., 2007). Studies indicate that ES

cells are transcriptionally permissive in a very general sense, with low level expression of lineage restricted genes and normally silent DNA repeats regions. The short, aborted products of this permissive state have been proposed to participate in gene silencing at specific loci, with studies implicating RNA based mechanisms in the targeting of histone modifying enzymes and chromatin silencing complexes to loci targeted for heterochromatic repression (Fisher & Fisher, 2011; Mattout & Meshorer, 2010).

Compared to pluripotent ES cells, the nuclei of differentiated cells contain numerous large areas of electron dense material associated with compact heterochromatin (Niwa, 2007; Meshorer & Misteli, 2006). By ChIP and immuno-fluorescence based assays, lineage specification is accompanied by a global decrease in levels of active histone marks (such as acetylated histone H3 and H4, H3K4me3) and an increase in repressive histone marks (such as histone H3K9me) (Azuara et al., 2006; Meshorer et al., 2006). Studies employing DamID and ChIP-chip analyses to compare ES cells and their differentiated counterparts demonstrate that differentiated cells relocalize pluripotency factors to the nuclear lamina, an area highly correlated with gene silencing, while areas associated with H3K9me increase in size and abundance in a cell type specific fashion (Peric-Hupkes et al., 2010; Wen et al., 2009). My work focuses on the identification of factors involved in chromatin compaction and transcriptional silencing during the transition from a developmentally plastic state to a lineage committed state, using the early *C.elegans* embryo as a model.

Polycomb and Pluripotency in *Drosophila* and mammals

Among the factors likely to function at the intersection of pluripotency and cell fate commitment are the Polycomb group (PcG) transcriptional repressors. Initially identified as playing a role in the regulation of anterior/posterior body patterning via repression of *Hox* genes in *Drosophila*, Polycomb factors are now widely appreciated to function in a range of processes including cell cycle control, genomic imprinting, X-inactivation, cell fate transitions, tissue homeostasis, and tumorigenesis (Surface et al., 2010).

Although many PcG proteins are well conserved in mammals, plants, *Drosophila* and *C.elegans*, the composition of the PcG complexes is likely to vary among different cell types in a context and perhaps organism specific manner. The PcG transcriptional repressors generally associate with either Polycomb Repressive Complex (PRC) 1 or 2. While these complexes are functionally distinct, both are thought to regulate chromatin structure. The PRC2 complex catalyzes the di and tri methylation of histone H3K27, and is composed of three core protein components in flies and mammals; the histone methyltransferase (HMT) Ezh2/E(Z), as well as Eed/Esc (WD40 domain) and Suz12 (Zn finger domain) (Schuettengruber et al., 2007; Surface et al., 2010; Simon & Kingston, 2009). The core PRC2 proteins interact with an ever expanding list of factors, including the generic histone binding proteins RbAp46/48 (also known as Rbbp7/4), an alternative HMT (EZH1), histone demethylases, such as Jarid2, AEBP2 (Zn finger), and several PCL (Polycomb like) proteins (Margueron & Reinberg, 2011). The diverse interaction partners have been proposed to refine the function of the core complex, regulate enzymatic activity, facilitate conversion between di to tri H3K27 methylation, define

tissue specificity, and/or direct recruitment to target promoters (Surface et al., 2010; Sauvageau and Sauvageau, 2010).

The core components of PRC1 complex include Pc (Polycomb), Ph (Polyhomeotic), Bmi1/Mel18, and the catalytic components Ring1A/B (Levine et al., 2002). PRC1 mediates the monoubiquitination of histone H2AK119 though the ubiquitin E3 ligase activity of RING1B, and may direct chromatin compaction independent of enzymatic activity (Francis et al., 2004; Eskeland et al., 2010). Additional complexes involved in PcG function have been identified in both *Drosophila* and mammals, including the Pho Repressive Complex (PhoRC) and Polycomb Repressive Deubiquitinase (PR-DUB) (Levine et al., 2002). Orthologs of these complexes, as well as the potentially regulatory subunits described above, remain to be identified in many species. Based on these data, however, one emerging hypothesis suggests that multiple versions of PRC1 and PRC2 exist, with mounting evidence that alternative subunit compositions may confer distinct target gene specificity.

PcG complexes (specifically PRC1 and PRC2) share a number of targets by genomewide analysis, including a large number of transcription factors, morphogens, receptors, and signaling proteins involved in major developmental pathways (Schwartz & Pirrotta, 2007). However, whether the PRC1 and PRC2 complexes function cooperatively to silence target loci is currently unresolved. PRC1 has been shown to bind directly to H3K27me3 via Pc, leading to the hypothesis that PRC1 functions downstream of PRC2 (Schwartz & Pirrotta, 2007); this model is schematized in Figure 2. In support of this view, disruption of PRC2 leads to loss of PRC1 at target loci in both *Drosophila*

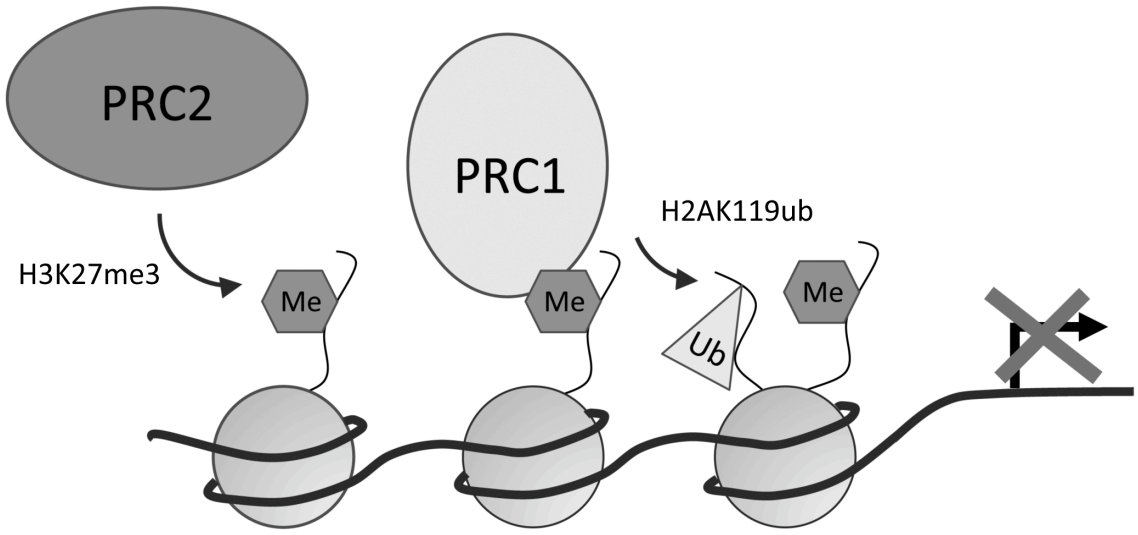


Figure 2. Controversial interaction between Polycomb repressive complexes. PRC2 mediates H3K27me3. PRC1 is thought to bind this methyl mark, induce ubiquitination of H2AK119 and initate chromatin compaction. However, various studies suggest that H3K27me3 may be recognized by alternative factors responsible for transcriptional silencing.

and mammalian cell culture (Wang et al., 2004; Cao et al., 2002; Cao et al., 2005; Boyer et al, 2006). Other studies supporting the idea that PRC1 function tracks with H3K27me marks employed H3K27 demethylation mutants and a viral methyltransferase, which both perturb the levels of H3K27me3 without affecting the PRC2 complex, yet induced changes in PRC1 targeting (Lee et al., 2007; Mujtaba et al., 2008). Moreover, chromatin decompaction is observed in Ring1B (PRC1) deficient cells despite the presence of PRC2 mediated H3K27me3 marks, suggesting that PRC1 is necessary to compact PRC2 target loci, and arguing against independent functions (Eskeland et al., 2010).

In contrast, genomewide ChIP-seq analyses indicate that PRC1/H2Aub bound sites overlap with PRC2/H3K27me3 at only a subset of silenced promoters, and demonstrate the binding capacity of PRC1 in the absence of PRC2 methylation (Ku et al.,

2008; Schwartz et al., 2006; Boyer et al., 2006; Leeb et al 2010, Schoeftner et al., 2006). For example, RING1B recruitment is independent of H3K27me2/3 and instead requires the presence of Xist at an early stage of X chromosome inactivation (Schoeftner et al., 2006). Studies have demonstrated that PRC1 remains bound at some target loci following depletion of H3K27me3 in fly and mouse, suggesting that PRC2 may not be required for PRC1 function (Nekrasov et al., 2007; Schoeftner et al., 2006; Lee et al., 2007). Lastly, the binding affinity between PRC1 and H3K27me3 is less than typical regulatory protein–DNA interactions, suggesting that although this binding is possible, it may not be probable *in vivo* (Schwartz & Pirrotta, 2007).

Data supporting independent functions of PRC2 and PRC1 are not mutually exclusive with data supporting cooperative silencing; the H3K27me3 mark is likely to configure local chromatin environments and interact with any number of additional protein complexes at target loci, at least one of which is PRC1 in flies and mammals. It has also been suggested that interactions between PRC1 and methylated H3K27 facilitate DNA looping and/or interactions between promoter elements and distal enhancers (Simon & Kingston, 2009). In plants and worms, where the existence and function of PRC1 like complexes are less clear, it has been suggested that a multi functional complex capable of H3K27 methylation, chromatin compaction, and potentially coordination of H3K4 demethylation and resolution of bivalent domains, might abrogate the need for a PRC1 like complex (Whitcomb et al., 2007; Simon & Kingston, 2009). Data indicating that chromatin compaction and gene silencing may not rely on the catalytic activities of PcG complexes adds to the mystery regarding how transcriptional silencing is achieved *in vivo* (Simon & Kingston, 2009; Eskeland et al., 2011).

Despite numerous recent studies, many unknowns remain to be examined regarding PcG proteins. The H3K27me3 mark is clearly correlated with transcriptional repression, yet the specific mechanism(s) by which this repression is accomplished remains elusive. While some histone tail modifications (i.e., H3K79, H4K20, and acetylation) have been shown to affect nucleosome-DNA interactions and higher-order chromatin structure, there is no evidence for H3K27me3 having these affects, and unlike PRC1, the core PRC2 complex does not compact nucleosome arrays *in vitro* (Simon & Kingston, 2009). In fact, very little is known regarding how the PRC2 complex interacts with chromatin, as core components do not contain recognizable DNA binding motifs (Simon & Kingston, 2009). Another unknown involves the localization of PcG complexes to specific target loci, although a number of methods of recruitment have been proposed, including Polycomb Responsive elements (PRE) in *Drosophila*, CpG islands in mammals, interactions with the PC-Rho complex, insulator sequences and directed DNA looping, as well as noncoding RNAs (ncRNAs) (Margueron & Reinburg, 2011).

Polycomb and pluripotency

Current models suggest that cellular plasticity is terminated when factors required for pluripotency are silenced, and/or regulators promoting differentiation are upregulated. Although the roles of PcG proteins in ES cells and pluripotency have been debated, studies suggest that PcG proteins are important to define cellular gene expression programs and ensure proper cell fate specification (Sauvageau and Sauvageau, 2010).

In pluripotent ES cells, a core network of transcription factors, including Oct4, Sox2 and Nanog are required to establish and maintain pluripotent cell potential, as loss

of these factors leads to spontaneous differentiation (Surface et al., 2010). Genome-wide ChIP data in ES cells demonstrate that significant overlap in genes repressed by OCT4 and/or Nanog and genes shown to be targets of PcG, indicating that PcG silencing might be recruited by and collaborate with these transcription factors to regulate gene expression in these cells (Lee et al., 2006; Bernstein et al., 2006; Boyer et al., 2006). In these cells, genes targeted for PcG repression include many key developmental regulators such as Fox, Wnt, Sox, Pou, Pax, GATA, and Tbx family members (Boyer et al., 2006; Ku et al., 2006; Lee et al., 2006). Developmental regulators lose H3K27me3 and become activated during ESC differentiation, and by FISH analysis, undergo local decompaction, consistent with increased transcriptional activity (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006; Stock et al., 2007; Tolhuis et al., 2006; Eskeland et al., 2010). Conflicting data have contributed to a highly controversial hypothesis suggesting that relief of PcG mediated repression may represent a key event in the transition from developmentally plastic to differentiated cell types.

Loss of core PRC2 component activity results in early embryonic lethality in mice, whereas inactivation of PRC1 components results in less severe phenotypes occurring later in development (Faust et al., 1995; Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004; Surface et al., 2010). Although previous studies suggested that PcG mutant ES cell lines could not be established, more recent studies indicate that ESCs lacking PRC function can be generated as well as maintained, suggesting that PcG proteins are not required for pluripotency (Surface et al., 2010; Margueron & Reinburg, 2011). For instance, *Suz12*(-/-) mouse ESC lines can be established and expanded in tissue culture, and are characterized by loss of H3K27me3 and overexpression of differentiation specific

genes (Pasini et al., 2007; Pasini et al., 2010). In addition, *Suz12*(-/-) ES cells are unable to contribute to endodermal lineages in chimeric implants (Pasini et al., 2007). Additional PcG mutant cell lines show similar impairments in differentiation potential and/or differentiate spontaneously, due to overexpression of differentiation specific genes and/or increased expression of pluripotency factors including OCT4 and Nanog, which normally cooccupy the promoters of PcG genes (Boyer et al., 2006; Chamberlain et al., 2008; Shen et al., 2008; Lee et al., 2006; van der Stoop et al., 2008). The inability of PcG mutant ESCs to maintain expression of lineage specific genes and differentiate into specific cell lineages strongly suggests that PcG proteins are essential for cell fate transitions.

It remains unclear what role PcG complex(es) play in the coordination of pluripotency and differentiation, as are the mechanisms of target loci derepression required to achieve lineagerestriction. The extent to which PcG factors control these processes remains controversial, and are further complicated by potential redundancy among the various subunits in some species. In *Drosophila* and plants, at least 15 PcG factors have been identified, while as many as 37 have been identified in mice and humans (Sauvageau and Sauvageau, 2010). Therefore, interpretation of phenotypic severity should be considered in light of possible functional and/or genetic redundancy.

Interestingly, only three components of PRC2 have been identified in *C.elegans*, providing us with a unique opportunity to investigate how different pathways interact with Polycomb mediated silencing during the transition from pluripotency to cell fate specificity. In this system, the Mango Lab has demonstrated a role for PcG factors during the transition from cellular plasticity and differentiation. We've shown that PcG

proteins function to repress pluripotency factors at the onset of differentiation, rather than repress developmental regualtors during pluripotency, as previous models might have suggested (Yuzyuk et al., 2006). As PcG factors may not be required for pluripotency in ES cells, this model may represent a general mechanism of Polycomb function. *C.elegans* PcG factors are discussed in detail in later sections.

Bivalent domains and cell fate restriction

Genomewide mapping data in ESCs have demonstrated that a number of developmental and tissue specific target genes are simultaneously marked with PcGmediated H3K27me3 (repressive) and Trithorax induced H3K4me3 marks, which correlate with active transcription. Many of these promoters are also bound by RNA PolII phosphorylated at Serine 5 of the C terminal domain (CTD), a mark associated with transcriptional initiation, and in this case may represent paused polymerase (Bernstein et al., 2006; Ku et al., 2008; Stock et al., 2007; Guenther et al., 2007). Termed "bivalent domains," this duo of PTMs correlates with silent or low level expression in ESCs, and is thought to represent genes that are poised for rapid activation following induction of differentiation (Stock et al., 2007; Azuara et al., 2006).

The resolution of the bivalent domains is thought to be related to cell fate commitment, although whether it is a driving force remains to be determined (Mikkelsen et al., 2007). Upon differentiation, H3K27me3 marks are erased from the promoters of activated genes, while H3K4me3 marks are maintained (Bernstein et al., 2006). Conversely, the promoters of genes destined to remain repressed in a given cell type retain the H3K27me3 while H3K4me3 is removed (Bernstein et al., 2006). Removal

of these marks have been linked to histone demethylases specific for H3K4me3 (i.e., LSD1), and H3K27me3 (i.e., JMJD3 and UTX), consistent with an essential role for these demethylases in embryonic development (Adamo et al., 2011; Lan et al., 2008; Lee et al., 2007; Xiang et al., 2007; Agger et al., 2007; Swigut & Wysocka, 2007). It has also been suggested that the PRC2 complex may aid in the targeting of H3K4 demethylase activity to specific loci to maintain gene repression during ES cell differentiation (Pasini et al., 2008). These findings are consistent with a model in which developmental expression profiles are coordinated by the opposing activities of PcG proteins and Trithorax factors.

Bivalent domains can be subdivided based on the presence of PRC2 or both PRC1 and PRC2, the latter of which more efficiently maintain H3K27me3 upon differentiation (Ku et al., 2008). In addition, bivalent domains whose promoters show broader coverage of H3K27me3 are more likely to be silenced following cell fate commitment (Hawkins et al., 2010). Some developmentally important loci, including the Oct4 promoter, are further silenced by the addition of H3K9me or DNA methylation following differentiation of ESCs; this redundancy may reinforce H3K27me3 silencing and ensure unidirectional differentiation (Hawkins et al., 2010).

One possible mechanism by which PcG repressors might inhibit gene activity at bivalent promoters involves inhibition of RNA polymerase elongation. Although the mechansims remain obscure, this pausing has been suggested to rely on the short abortive transcripts synthesized from these promoters (Guenther et al., 2007; Bernstein et al., 2006, Boyer et al., 2006, Lee et al., 2006). Recent ChIP-Seq and global runon sequencing (GRO-Seq) based studies have linked Polycomb with inhibition of RNA PolII

recruitment to bivalent promoters by demonstrating that promoter association of Pol II is significantly increased in Esc mutants (Chopra et al., 2011; Min et al., 2011). Similar to previous studies, a greater stringency of inhibition is observed when both PRC2 and PRC1 bound to a bivalent promoter (Min et al., 2011).

Bivalent domains are not a unique feature of pluripotent cells but are also present in differentiated cell types in culture, as well as in early zebrafish embryos (Azuara et al., 2006; Mikkelsen et al., 2007; Vasthenow et al., 2010). During zebrafish embryogenesis, bivalent domains are formed at the onset of zygotic transcription; Vasthenow and collegues associate this period with embryonic pluripotency similar to what we have observed in *C.elegans*.

Transcriptional silencing, cellular plasticity, and the MES pathway in *C.elegans*

Cellular plasticity and cell fate commitment in *C.elegans*

The nematode *C.elegans* is increasingly appreciated as a powerful model to study developmental plasticity within an intact embryo. In *C.elegans,* specific cellular characteristics can be observed as early are the two cell stage of embryogenesis, a finding that was initially interpreted as very early cell acquisition of cell fates (Sulston et al., 1983; Gonczy & Rose, 2005). However, multiple lines of evidence indicate that the somatic blastomeres are developmentally plastic until the 2E (for 2 endodermal cells) stage of embryogenesis, with a transition to cell fate commitment beginning at the 8E stage.

The first piece of evidence supporting early developmental plasticity comes from analysis of the *C.elegans* lineage. Prior to the onset of gastrulation, most blastomeres contribute to multiple cell types, whereas after the 8E stage, decendants of a cell typically contribute to a single organ or tissue, suggesting that cells commit to a specific lineage around 8E (Sulston et al., 1983). Second, early blastomeres can adopt alternative fates when developmental transcription factors are ubiquitously expressed under the control of a heatshock promoter (Fukushige and Krause, 2005; Gilleard and McGhee, 2001; Horner et al., 1998; Kiefer et al., 2007; Smith and Mango, 2007; Zhu et al., 1998; Yuzyuk et al., 2009). Prior to 2E, this "cell fate challenge" will result in dramatic, nearly embryowide conversion to the alternate cell fate, but this response is severely reduced after the 8E stage, indicating that many cells have become committed. Third, blastomere exchange experiments performed prior to 2E demonstrate that a subset of cells are able to adopt different fates when moved to a new position within the embryo, demonstrating flexibility in response to intracellular signaling (Wood, 1991; Shelton & Bowerman, 1996). While the *C.elegans* lineage is invariant, this developmental flexibility indicates that the worm does not rely on predetermined patterns of development as previously thought, but instead responds to reproducible patterns cell signaling. The fourth piece of evidence supporting the plasticity of early blastomeres involves the mutation of early functioning regulatory transcription factors. Loss of transcription factors functioning prior to 8E often induce cell fate transformations, while perturbation of post-8E functioning transcription factors have more subtle phenotypes (Mango, 2007). By expression analysis, dramatic changes in transcript pools coincide with the observed remodeling of nuclear morphology, consistent with global changes in cellular identity

(Yuzyuk et al., 2009; Baugh et al., 2003). These global changes in transcription coincide with the onset of gastrulation, and may reflect a switch from maternal to bulk zygotic transcription (Yuzyuk et al., 2009; Baugh et al., 2003). Based on these data, the transition phase of *C.elegans* embryonic development may be analogous to the midblastula transition (MBT) observed in other animals. MBT represents a major turning point in the life of an embryo, involving a dramatic upregulation of zygotic transcription, changes in the cell cycle and cell division dynamics, and increased cell movement (Heasman, 2006; Edgar & McGhee, 1988; Sulston et al., 1983; Etkin, 1988).

Changes in nuclear architecture similar to those observed in mammalian ES cells have been observed in the nuclei of *C.elegans* embryos during the 2E (plastic) to 8E (committed) transition (Yuzyuk et al., 2009; David Hall, pers. communication) suggesting that the remodeling of nuclear architecture is a key feature in the onset of cell fate commitment. In sum, the 2E-8E transition period in characterized by changes in blastomere plasticity, nuclear architecture, and transcriptional expression.

Transcriptional silencing in the early *C.elegans* germ line

Embryonic cells are born with the ability to become a diverse number of cell types, and are thus plastic in nature. In order to generate a functional organism capable of reproduction, these pluripotent cells must proliferate, commit to specific cell fates, and undergo differentiation while preserving the pluripotent compartment (i.e., the germ line), which gives rise to the next generation (Strome, 2005). The germ line and somatic tissues of multicellular eukaryotes are functionally distinct. The germ line remains pluripotent and maintains reproductive capacity necessary to give rise to subsequent

generations, while somatic cells derived from the germ line give rise to all other cell types and tissues required to generate a function animal. The pluripotent germ line must be insulated from somatic signals in order to preserve reproductive potential.

Transcriptional repression is a necessary feature of the germ line in flies and *C.elegans*, as loss of silencing mechanisms induces failure in germ cell formation and survival (Strome, 2005). In the *C.elegans* embryo, the germ line is generated through a series of asymmetric cell divisions to produce the germ line blastomere (P4)(Strome, 2005). Transcription is inhibited in P4 by maternally endowed PIE-1, which inhibits transcriptional elongation by blocking phosphorylation of the RNAP II C-terminal domain (CTD) (Strome, 2005). P4 divides only once during embryogenesis, producing the primordial germ cells (PGCs) Z2 and Z3 at ~100 cell stage (Strome, 2005). PIE-1 is degraded in these cells, and RNA PolII is transiently activated, evidenced by CTD phosphorylation (Strome, 2005). Following this window of transcriptional activation, PGCs remain transcriptionally silent until the embryo is hatched and germ line proliferation begins.

This second phase of transcriptional inactivity is PIE-1 independent, and may rely on a chromatin based mechanism, as marks associated with gene activity, including H3K4me and H4acetylK8 are absent in these cells, while the repressive mark H3K27me2/3 is retained and/or enriched (Schaner et al., 2003; Bender et al., 2004). PGC DNA has been shown to maintain the decompact architecture associated with pluripotent cells (Schaner et al., 2003). It is currently unclear how repression is maintained in the PGCs, although the MES factors (discussed in detail in the following sections) likely play a key role.

Maternal effect sterile (*mes*) factors

Similar to orthologs in other species, the Polycomb group of transcriptional repressors play important roles in *C.elegans* development, including inactivation of the X chromosomes, and silencing of transposons, multicopy transgenic elements, and developmental regulators. PcG/Mes factors also contribute to germ line/soma cell fate distinctions and the coordination of pluripotency termination. The following sections describe the MES factors, including the *C.elegans* PRC2 complex (MES-2, MES-3, MES-6), antagonism of PRC2 by MES-4, and the roles of these proteins in the control of chromatin dynamics during development.

The maternal effect sterile (*mes*) genes were identified in a screen for "grandchildless" mutants (Capowski et al., 1991). These factors are maternally donated and function in germ line development. Four *mes* genes, *mes-2*, *mes-3*, *mes-4*, and *mes-6* have strikingly similar phenotypes with respect to the germ line. Loss of these genes result in fertile hermaphrodites (M+Z-, for maternal (M) and zygotyic (Z)) whose offspring (M-Z-) undergo reduced proliferation of the germ line coupled with necrotic degradation of the germ nuclei, resulting in a dramatic reduction in germ cell number and strict maternal effect sterility (Capowski et al., 1991; Paulsen et al., 1995; Holderman et al., 1998; Korf et al., 1998). The few germ cells that are visible in *mes* M-Z- animals, as well as in a proportion of M+Z- animals (i.e., ~15% in *mes-3*), display abnormal morphology and appear enlarged or swollen, with germline cytoplasm that is granular or coagulated in appearance, and contains large vacuoles and particles in Brownian motion (Capowski et al 1991, Paulsen et al., 1995; Garvin et al., 1998).

These defects are the result of germ cell degeneration and necrotic cell death, as the codepletion of programmed cell death factors (*ced-3*, *ced-4*) do not suppress the *mes* (-) germ cell degradation phenotype (Paulsen et al., 1995; Garvin et al., 1998). Dying germ nuclei also lack the characteristic "button" phenotype associated with apoptosis (Paulsen et al., 1995; Garvin et al., 1998). The failure of these mutants to produce functional gametes is not attributable to mitotic defects, aneuploidy, or the inability to respond to differentiation signals (Paulsen et al., 1995; Garvin et al., 1998). These data indicate that MES function is essential for proliferation of the germ line as well as for maintenance of viable germ cells competent to undergo gametogenesis.

Mes mutants are sensitive to changes in X-linked expression, as more severe loss of function phenotypes are associated with XXX animals than XX animals (Garvin et al., 1998). In contrast, germ cell degeneration is less apparent in XO animals (genetically "male"), which undergo gamete differentiation and retain fertility in a sex independent manner (Garvin et al., 1998). These observations led to studies demonstrating that X chromosomes are globally repressed in the germ line in a MES dependent matter (Kelly et al., 2002; Reinke et al., 2000; Fong et al., 2002; Bender et al., 2006; Bender et al., 2004; Kolasinska-Zwierz et al., 2009). Based on these findings, the primary function of the MES factors (PRC2 and MES-4) is thought to involve silencing the X chromosome the germ line, repression of which are necessary for germ cell viability.

There are currently two models of MES/PcG function. In the first, MES factors directly contribute to higher order chromatin structure through modification of histones. Alternatively, MES factors might inhibit function more locally at individual genes to transcription factors or interactions between enhancer elements and promoters. Animals

lacking maternal MES function are able to specify the germ line, but fail to proliferate (Capowski et al., 1991; Paulsen et al., 1998). As maternally donated MES protein is sufficient for fertility in M+Z- animals, MES/PcG proteins may function to establish, but not maintain chromatin structure inherited by the PGCs (Fong et al., 2002). The observed failure to proliferate and the propensity to degenerate have been suggested to result from inappropriate levels or patterns of gene expression, specifically with regard to the X chromosomes.

Germ line and embryonic expression patterns of MES factors

The MES proteins exhibit similar nuclear localization patterns during worm development. As predicted by the germ line phenotypes and maternal effect sterility, MES factors localize to adult germ line nuclei, are maternally donated and localized to oocyte nucleoplasm, and initially found in all blastomeres of the developing embryo (Xu et al., 2001; Holderman et al., 1998; Korf et al., 1998). At the ~100 cell stage, MES proteins begin to fade from the somatic lineage, and by the end of embryogenesis, are restricted to the primordial germ cells Z2/Z3 (Xu et al., 2001; Holderman et al., 1998; Korf et al., 1998; Fong et al., 2002). Germ line enrichment of MES factors is evident is larval stages through adulthood, where expression is highest in the mitotic and late pachytene regions, with marked reduction in the transition to midpachytene zones (Xu et al., 2001; Holderman et al., 1998; Korf et al., 1998; Fong et al., 2002).

Although the *mes* factors have similar localization patterns and loss of function phenotypes, they appear to function in two separate complexes. MES-2, MES-3, and MES-6 form a complex analogous to Polycomb repressor complex (PRC2), and mediate

transcriptional repression by methylation of H3K27 (Xu et al., 2001; Bender et al., 2004). MES-4 contains a SET domain (Suppressor of variegation, Enhancer of zeste, Trithorax; a domain defining a large family of histone lysine methytransferases) and three PHD fingers (Plant Homeo Domain, mediating protein-protein interactions) (Bender et al., 2006). MES-4 is homologous to mouse NSD1 (Nuclear receptor binding SET Domain 1) and is required for H3K36me (Bender et al., 2006). Current models suggest that MES4 functions antagonistically to the MES/PRC2 complex, specifically with respect to silencing of the X chromosome.

MES-2/MES-3/MES-6 complex: Orthologous to PRC2

MES-2 and MES-6, along with the novel factor MES-3, function in a complex in the worm orthologous to PRC2 (Xu et al 2001; Korf et al., 1998). MES-2, a SET domain protein homologous to *Drosophila* Enhancer of Zeste E(Z) and human EZH2, is the catalytic component of this complex, di and tri methylating H3K27 *in vitro* and *in vivo* (Bender et al., 2004; Strome, 2005). MES-6 is a WD repeat containing protein homologous to *Drosophila* Extra Sex Combs (Esc) and human Eed (Strome, 2005). MES-2 and MES-6 interact with MES-3, a novel protein containing no known functional domains (Paulsen et al., 1998).

Proper nuclear localization of the PRC2 complex members is interdependent, consistent with PcG factors in other organisms (Xu et al., 2001; Rastelli et al., 1993; Platero et al., 1996). MES-2 and MES-6 localization is mutually dependent, while proper localization of MES-3 requires both MES-2 and MES-6 during mid-stage embryogenesis, but not during early embryogenesis or germ line development (Xu et al.,

2001). This suggests that MES-3 may not function with MES-2 and MES-6 in all contexts, and may be replaced by an alternative subunit in some scenarios (Xu et al., 2001). In addition, *mes-3* mutations have somewhat intermediate effects on the localization of MES-2 and MES-6 (Holderman et al., 1998; Korf et al., 1998; Xu et al., 2001).

The *C.elegans* PRC2 complex is similar to that observed in other species, with respect to the contribution of noncatalytic subunits to overall HMT activity (Ketel et al., 2005). *In vitro*, the complete ESC-E(Z) complex, containing Suz-12 and Nurf55, is 1,000 fold more active than E(Z) alone (Ketel et al., 2005). In the absence of MES-3 and MES-6, MES-2 in the absence of other components lacks HMT activity *in vitro*, as do the MES-2/MES-3 and MES-2/MES-6 dimers (Ketel et al., 2005). MES-2 is required for complex formation, as MES-3/MES6 do not dimerize *in vitro* (Ketel et al., 2005). Therefore, MES-2 binding to both MES-3 and MES-6 is required to generate robust HMT activity, indicating that loss of any of the components will result in loss of PRC2 activity *in vivo*, a finding consistent with loss of H3K27me marks in different RNAi treatments (Bender et al., 2004; Senchuk et al., unpublished). A previous study found that MES-3 does not directly interact with either MES-6 or MES-2 (Xu et al., 2001), which suggests that additional factors may mediate the formation of the complete complex, or that both MES-2 and MES-6 are required for optimal binding of MES-3.

The MES/PRC2 complex has not been extensively purified from worm extracts, therefore, it is possible that additional subunits remain to be identified. In coIP experiments, MES-6 pulls down only MES-2 and MES-3 (Bender et al., 2004), indicating that these factors may represent the sole members of the complex, or may function as the

core complex, with additional/alternative subunits binding more transiently, weakly, or in a context dependent manner, as has been observed in other species. The MES/PRC2 complex immunoprecipitated from *C.elegans* embryo extracts is able to methylate nucleosomes but not free histones, which are targeted by the cognate complex isolated from *Drosophila* extracts (Bender et al., 2004; Ketel et al., 2005). This may indicate the requirement of additional cofactors, or may indicate that MES/PRC2 function is somewhat more specific than PRC2 in other species, perhaps mediated by the novel MES-3. Data from sucrose density gradient and gel filtration analyses are also somewhat unclear as to whether MES-2/3/6 are the sole components of this complex, the predicted size of which is 230 kDa. The experimental weight of the complex is 255 kDa (Xu et al., 2001); thus the interaction of an additional small protein or posttranslational modification cannot be ruled out. More than 15 PcG components have been identified in the fly, and while orthologs of these components have not been identified in *C.elegans* by sequence homology, functional homology has not been fully explored.

While the MES/PRC2 complex is localized to the nucleus, MES-2, MES-3, and MES-6 do not appear to localize specifically to chromatin (Holderman et al., 1998; Korf et al., 1998; Xu et al., 2001). Inferences about the activity and targeting of this complex are primarily based on immunofluorescence based analysis of MES/PRC2 dependent H3K27me, which have demonstrated enrichment on X chromosomes in the germ line, supporting the hypothesis that MES proteins function in direct transcriptional repression of the X chromosome (Bender et al., 2004).

Immunofluorescence assays have also been used to examine the distinct patterns of MES/PRC2 mediated di and trimethylation during development. In the germ line and

early embryo, H3K27me2 is localized all chromosomes in all nuclei, with a nonuniform or "banded" pattern of chromatin staining (Bender et al., 2004). Just prior to the division of the Z2/Z3 PGCs (~100 cell stage), H3K27me2 is decreased in the P4 germ line blastomere, and remains undetectable in the germ lineage through embryogenesis (Bender et al., 2004). In contrast, H3K27me3 is highly enriched on the X chromosomes in both germ line and embryonic nuclei (Bender et al., 2004). As observed for H3K27me2, H3K27me3 is present in all embryonic nuclei until the 100 cell stage, but when H3K27me2 decreases in the PGCs (Z2/Z3), H3K27me3 becomes enriched in these cells, consistent with the global repression of the germ line (Bender et al., 2004; Strome, 2005). These findings generally reflect the expression pattern of MES-2/MES-3/MES-6, and suggest that H3K27me3, rather than H3K27me2, represents the primary repressive mark in the germ line precursor cells.

Analyses of H3K27me patterns in *mes-2*(-) animals has demonstrated that MES-2/PRC2 is responsible for the majority of H3K27me2 and H3K27me3 in most of the germ line and early embryogenesis (Bender et al., 2004). Depletion of *mes-2/3/6* leads to loss of H3K27me2 and H3K27me3 in the distal germ line, although some accumulation in seen in late pachytene and oogenesis (Bender et al., 2004). These marks might be attributed to an as yet unidentified HMT functional in the proximal germ line. However, these methyl marks are absent in early embryos, with the accumulated signal being extruded with the meiotic polar body (Bender et al., 2004).

At least one additional non-MES HMT dimethylates H3K27 during embryogenesis, as this mark is observed in *mes* (-) embryos, allowing for near wildtype levels in some embryonic nuclei and relatively normal levels of H3K27me2 throughout

the somatic tissues by adulthood (Bender et al., 2004). In addition, this or yet another HMT capable of catalyzing the dimethyl mark becomes active specifically in the PGCs of *mes*(-) mutants at the 100 cell stage (Bender et al., 2004). This mark appears elevated in *mes-2* mutants compared to wildtype embryos, suggesting a failure in the conversion of di to trimethyl (Bender et al., 2004). As H3K27me3 remains low in all somatic tissues as well as the PGCs throughout development in *mes-2(-)*, it is possible that MES/PRC2 function is unnecessary for H3K27me2 in the PGCs, but is required for H3K27me3. Bender and colleagues hypothesized that in PGCs, the MES/PRC2 complex may prevent accumulation H3K27me2 by blocking expression or activity of the dimethylase, or by rapid conversion of H3K27me2 to H3K27me3. Similar staining patterns were observed in the germ cell nuclei of males, indicating that MES function is not hermaphrodite specific (Bender et al., 2004).

In addition to H3K27 methylation, MES/PRC2 function may also be required for the majority of H3K9me3 in the germ line, although this complex appears to be dispensable for H3K9me2 (Bessler et al., 2010). Immunofluorescence data in this study differ from findings of similar assays, as well as *in vitro* data published previously, possibly reflecting the use of different antibodies and/or more careful analysis of the germ line compared to embryos (Bender et al., 2004; Andersen & Horvitz, 2007). *In vitro* biochemical studies of PRC2 are also inconclusive with respect to MES-2/E(Z)/EZH2 methylation of H3K9me3 (Muller et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002). These conflicting data indicate that H3K9me3 by PRC2 may be indirect, mediated by additional proteins, and/or occur in a spatiotemporally restricted manner. Even within the germ line, a subset of H3K9 methyl marks are MES-2

independent; H3K9me3 (like H3K27me3) is present on condensed chromosomes in diplotene and diakinesis, as well as within the mitotic zone, indicating the presence of at least one additional HMT capable of catalyzing H3K9me3 and H3K27me2/3 (Bessler et al., 2010).

An additional factor found to play a significant role in gene silencing in many systems is the HMT Su(Var)3-9, required for H3K9me2/3 (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001). Although *C.elegans* has no clear Su(Var)3-9 homolog, the SETDB1-like HMT MET-2 has been shown to methylate H3K9me2 and is required for normal levels of H3K9me2/3 in this species (Bessler et al., 2010; Andersen & Horvitz, 2007). By western blot, H3K9me3 levels are reduced as much as 80% in *met-2* mutant embryos (Andersen & Horvitz, 2007). Conversely, an immunofluorescense based analysis found that MET-2 is required for H3K9me2 but dispensable for H3K9me3 in adult germ cells (Bessler et al., 2010). While it is possible that the observed decrease in H3K9me3 in embryos was the result of decreased H3K9me2, these findings are not mutually exclusive, but suggest that the mechanisms of H3K9me3 differ in the germ line and somatic tissues. However, additional possibilities cannot be ruled out. For instance, the inefficiency or modulation of demethylase enzymes may account for residual methyl marks following loss of HMT function. There is also a precedent for a SETDB1 mediation of different marks in different tissues in *Drosophila*, lending further complication to interpretation of these findings (Bessler et al., 2010). Alternatively, additional HMT(s), including MES-2, may be responsible for the majority of H3K9me3 during development. However, H3K9me3 is present in embryonic cells where MES-2 is not localized, suggesting the presence of additional HMTs, increased activity of MET-2

in mediating H3K9me3 in late embryogenesis, and/or the down regulation of demethylase activity in these cells, allowing purdurance of H3K9me3.

Intriguingly, *mes-2; met-2* double loss of function did not demonstrate additive phenotypes with respect to H3K9me marks and germ line function, indicating that these proteins are unlikely to be functionally redundant (Bessler et al., 2010). Despite mediating the similar marks, some data suggest that *mes-2* and *met-2* may have opposing functions, as opposite phenotypes are observed with respect to suppression/enhancement of a strain carrying mutations in each of the partially redundant heterochromatin 1 (HP1) factors, *hpl-1; hpl-2*. *mes-2* suppresses the *hpl-1; hpl-2* larval arrest phenotype, while *met-2* enhances this phenotype, inducing larval arrest and lethality at an earlier stage (Simonet et al., 2007; Senchuk et al., unpublished). Based on these data, MES/PRC2 dependent repression likely involves H3K27me2/3 and H3K9me3, while the HMT MET-2 mediates H3K9me2. It is currently unclear how these factors interact.

By chromatin immunoprecipitation (ChIP) analysis, H3K27me2/3 marks across the *C.elegans* genome are anticorrelated with levels of gene expression, RNA PolII localization, and marks associated with gene activity, consistent with a role in transcriptional repression (Liu et al., 2011). The H3K9me2/3 is correlated with gene repression in other systems, and ChIP data in *C.elegans* indicate that H3K9me2/3 marks are enriched on silent genes and repetitive sequences, and are found associated with nuclear lamina proteins, suggesting these marks may denote more stably silenced heterochromatin associated with the nuclear periphery (Liu et al., 2011; Gerstein et al., 2010).

One of the primary domains associated with MES/PRC2 repression are the X chromosomes. Immunofluorescence analyses have demonstrated enrichment of MES dependent H3K27me3 throughout the proliferative (mitotic) and most of the maturing (meiotic) germ line in both hermaphrodites and males (Bender et al., 2004). In addition to enrichment of H3K27me3, X chromosomes lack activating marks including H3K4me2, H3K36me2, histone acetylation, and RNA PolII CTD Serine 2 phosphorylation, a mark associated with transcriptional elongation (Kelly et al., 2002; Reuben and Lin, 2002). These data are consistent with very low expression of X-linked genes in the germ line of either sex, consistent with the paucity of germ line genes encoded on the X chromosome (Reinke et al., 2000; Bender et al., 2006). Mutation of *mes-2*, *mes-3*, or *mes-6* leads to inappropriate acquisition of active marks, demonstrating that MES/PRC2 proteins are required for the silencing of X chromosomes (Fong et al., 2002; Strome, 2005). Silencing of the X chromosomes and transgenic elements also requires MES-4 (Bender et al., 2006; Kelly et al., 1998).

Xu and colleagues (2001) proposed that the MES system functions in the maternal germ line and early embryos to epigenetically mark genes and regulate expression in the embryonic germ line (Z2/Z3), suggesting that somatic genes, and the X, which contains few germline genes, are marked for transcriptional repression by H3K27me3. Following the degradation of PIE-1, germ line genes (i.e., not marked by H3K27me) become expressed. It is suggested that the desilencing of X encoded genes leads to the observed defects of germ cell degeneration and sterility in the F_1 generation. This idea is supported by the observation that marks of gene activation localize to the X chromosome in the

absence of MES-2/MES-3/MES-6 and that X chromosome dosage is an important determinant of lethality in *mes* mutants (Fong et al., 2002; Garvin et al., 1998).

MES-4: Antagonism of MES/PRC2

MES-4 is a histone methyltransferase that catalyzes di and trimethylation of histone H3 on lysine 36 (H3K36me2/3), marks correlated with gene expression in a number of systems including *C.elegans* (Lui, et al., 2011; Rechsteiner et al., 2010; Bender et al., 2006). MES-4 HMT activity appears to be specific for H3K36; additional histone marks, including H3K4me2/3, H3K9me2, H3K27me2/3, H3K79me2, and H4K20me2 are unaffected by the loss of *mes-4* (Fong et al., 2002; Bender et al., 2006). Similar to its homologs NSD1 (mouse) and MMSET (human), MES-4 contains three plant homeodomain (PHD) fingers, which mediate protein-protein interactions, and a SET domain flanked by cysteine rich regions required for HMT function (Fong et al., 2002).

Unlike MES/PRC2, MES-4 physically associates with chromatin via its first PHD domain (Fong et al., 2002; Bender et al., 2004). In contrast with the X localized PRC2 complex, MES-4 is enriched on autosomes and is excluded from the X chromosome in the germ line and early embryos, a finding consistent with ChIP data indicating very few MES-4 bound sites on the X chromosome (Fong et al., 2002; Bender et al., 2004; Rechsteiner et al., 2010). Based on immunofluorescence localization and biochemical assays, MES-4 does not associate with the MES/PRC2 complex (Fong et al., 2002; Bender et al., 2004). Instead, autosomal localization of MES-4 and H3K36me2/3 is dependent on the PRC2 complex and/or H3K27me2/3, as MES-4 spreads to the X

chromosome in MES/PRC2 mutants (Fong et al., 2002; Bender et al., 2004). Despite the observed autosomal localization and correlation of H3K36 methylation with gene activity, microarray data indicate that the primary effect of loss of MES-4 function is upregulation of X-linked genes, demonstrating that MES-4 participates in repressing genes on the X chromosome, a function similar to MES/PRC2 (Bender et al., 2006; Rechtsteiner et al., 2010).

It has been suggested that MES/PRC2 dependent H3K27me2/3 marks on the X chromosomes inhibit MES-4 binding and/or methylation of H3K36 (Bender et al., 2006). As depicted in Figure 3, two models have been proposed to account for the role of MES-4 in X-linked repression. In the first model, MES-4 might activate the expression of autosomal gene that induces repression of X-linked genes, possibly by influencing the localization of MES/PRC2. Alternatively, MES-4/H36me localized to autosomes might repel a limited repressor, such that repressive activity is concentrated on the X chromosomes. MES-4 antagonism is likely indirect, as patterns of H3K27me2/3 appear unchanged in *mes-4* mutants (Fong et al., 2002; Bender et al., 2006).

MES-4 is associated with, but not required for, expression in the germ line. This is evidenced by the lack of MES-4/H3K36me localization to the X during the transient period of X-linked gene expression at the end of pachytene (Fong et al., 2002; Bender et al., 2006; Kelly et al., 2002). During this time, the X chromosomes in hermaphrodites accumulate H3K4me2 and elongating PolII, likely involved in the transcription of oogenesis genes located on the X chromosome (Kelly et al., 2002).

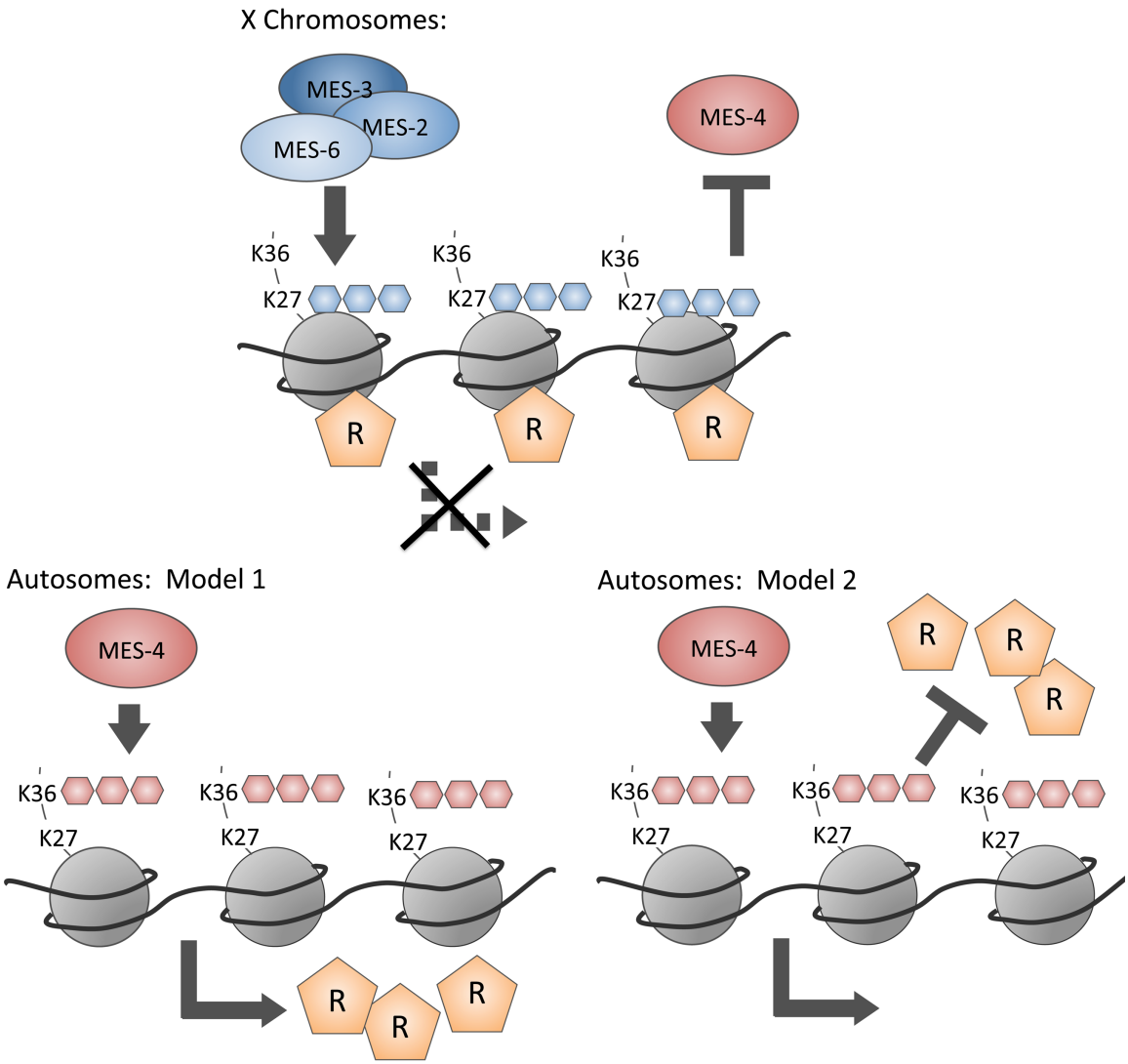


Figure 3. Schematic representation of current models of MES antagonism. The Mes/PRC2 complex mediates H3K27 trimethylation on the X chromosome. This mark and/or the localization of a repressor molecule (R), induces transcriptional repression and/or exclusion of MES-4. If such a repressor molecule exists, it is unknown how this factor interacts with H3K27me3. For instance, it may localize to the X chromosome by sequence specific binding or be recruited by/bind H3K27me3. As X enrichment of H3K27me3 is MES-4 independent, it is unlikely that the MES-4 associated repressor recruits PRC2. In the first autosome model, a repressor molecule (R) is transcribed from the autosomes in a MES-4/H3K36me3 dependent manner. In the second model, MES-4/H3K36me3 actively repels the repressive function, leading to concentration of repressive function on the X chromosome. These models are not mutually exclusive, but the second model predicts that MES-4 is not required for the production of R, and loss of MES-4 would result in repression of autosomes if R is not in limited supply. Adapted from Bender et al., 2006.

Silencing of the unpaired X chromosome and repetitive arrays

In *C.elegans*, extrachromosomal arrays are formed by the concatamerization of DNAs injected into the germ line syncytium, resulting in highly repetitive arrays carrying several hundred copies of a construct (Stinchcomb et al., 1985). In spite of the high copy number, expression of repetitive arrays is often lower than that observed from the endogenous locus, suggesting that these arrays are subject to silencing mechanisms (Mello & Fire 1995). Moreover, these arrays of often complete silenced in the germ line (Mello & Fire, 1995; Kelly et al., 1997). Germ line repression can often be relieved by coinjection with complex DNA (i.e., fragmented genomic DNA or exogenous DNA), resulting in 10-100 fold dilution of the expression construct (Kelly & Fire, 1998).

The MES proteins (-2, -3, -4, & -6) are required for the silencing of repetitive arrays in the adult germ line, a function consistent with the ascribed role of Polycomb in context and repeat dependent silencing in *Drosophila* (Kelly & Fire, 1998; Kennison, 1995; Pal-Bhadra et al., 1997). In *mes* mutants, germline desilencing occurs prior to cytological defects (i.e., necrotic degeneration of germ nuclei), lending support to the hypothesis that the primary function of MES factors in the germ line involves repression of target loci, including the X chromosomes and repetitive elements (Kelly et al., 1998). Kelly and colleagues also found that germ line degeneration is not indicative of germ line depression in most cases, as only 1/17 germ line defective mutants demonstrated desilenced transgene expression (Kelly et al., 1998). In addition to the MES factors, germ line silencing of high copy also requires a number of chromatin factors, including HPL-2/HP1, HIS-24/H1.1, and a subset of the conserved repressors known as SynMuv factors (Couteau et al., 2002; Jedrusik & Schulze, 2001; Kim et al., 2005; Cui et al.,

2006). Based on these findings, it's likely that silencing of repetitive transgenes in the germ line occurs at the level of chromatin.

A recent study demonstrated that different mechanisms are employed within the germ line to silence arrays carrying somatic specific promoters and arrays composed to germ line competent promoters. High copy arrays with somatic promoters are marked by H3K9me3, and do not appear enriched for H3K27me3 (Bessler et al., 2010). This result was observed even when the repetitive array was integrated into the genome, including sites on the X chromosome (Bessler et al., 2010). H3K9me3 also correlate with DAPI faint chromatin domains, suggesting these arrays may not be fully heterochromatinized (Bessler et al., 2010). Intriguingly, H3K9me3 enrichment on these arrays was also observed within the nuclei of somatic cells, suggesting that this mark does fully induce gene repression, or may reflect partial silencing, consistent with low level expression observed from high copy arrays (Bessler et al., 2010; Mello & Fire, 1995). Bessler & colleagues found that H3K9me3 is reduced, but not absent on these arrays in *mes-2* mutants, suggesting the presence of a MES-2-independent H3K9 methylation pathway. Alternatively, H3K9me3 may perdure at these sites in *mes-2* mutants due to inefficient demethylase activity. In the soma, high copy arrays are also marked with H3K27me3 (Towbin et al., 2010; Yuzyuk et al., 2009). In contrast to somatic-specific arrays, arrays bearing germ line competent promoters are enriched for H3K9me2, rather than H3K9me3 (Bessler et al., 2010; Kelly et al., 2002). Curiously, while H3K9me2 marks were depleted from germ line competent arrays in the absence of functional *met-2* HMT, desilencing of the array in the germ line was not observed (Bessler et al., 2010). It is currently unclear how MES/PRC2, which appears not to

methylate these arrays, contributes to maintenance of the silenced state within the germ line. However, this study did not examine H3K27me2/3, although previous studies suggest that these marks would be present and likely to change in *mes* loss of function (Towbin et al., 2010; Yuzyuk et al., 2009). Similarly, the role of MES-4 in this process is unknown, but it has been observed that MES-4 localizes to complex arrays (similar to autosomes), and is excluded from repetitive arrays (Fong et al., 2002), suggesting that mechanisms of array silencing are likely similar to X chromosome silencing, or may be due to lack of activating marks, rather than enrichment of silencing marks. These findings highlight the complex dynamics of histone modifications in the regulation of chromatin activity.

Similar to somatic specific promoter arrays, the unpaired male X chromosome, as well as free autosomal duplications, are enriched for H3K9me2 (Kelly et al., 2002; Bean et al., 2004). The lone X is also highly condensed and lacks marks of transcriptionally competent chromatin throughout the adult germ line, including H3K4me2 (Kelly et al., 2002; Bean et al., 2004). This meiotic silencing may involve siRNA based mechanisms, as the RNA directed RNA polymerase (RdRP, involved in amplifying dsRNA) EGO-1 is required for H3K9me2 enrichment (Maine et al., 2005). CSR-1 (an Argonaute protein), DRH-3 (a Dicer-related DEAH/D-box helicase), and EKL-1 (a Tudor domain protein) all, like EGO-1, promote RNAi and influence meiotic H3K9me2 distribution (She et al., 2009). Loss of MET-2 HMT activity, and presumably H3K9me2 marks, leads to ectopic deposition of H3K4me activation marks on the unpaired X (Checchi & Engebrecht, 2011). *mes-2* and *met-2* depletion result in similar increases germ line apoptosis

phenotypes in XO germ lines, suggesting that MES-2 activity is also required for silencing of the unpaired X (Checchi & Engebrecht, 2011).

MES interactions with synthetic multivulva (SynMuv) factors

While MES-4 activity appears to oppose MES/PRC2, a group of proteins collectively known as SynMuv factors likely oppose MES-4 function. The SynMuv genes (for Synthetic Multi-Vulva) were initially identified as redundant negative regulators of vulval differentiation, as loss of Class B SynMuv function coupled with loss of a Class A or C SynMuv gene function results in ectopic induction of vulval tissues and formation of multiple vulval structures (Muv), a phenotype that can be suppressed by mutations in the RAS signaling pathway (Cui & Han, 2007). The SynMuv factors promote somatic cell fate decisions in a number of developmental contexts.

SynMuv factors are highly conserved, chromatin associated proteins increasingly implicated in transcriptional repression. SynMuv factors include members of the nucleosome remodeling and histone deacetylase complex (NuRD), composed of MEP-1/HDA-1/LET-418, the heterochromatin 1 (HP1) homolog HPL-2, the E2F components EFL-1 and DPL-1, and the retinoblastoma (Rb) homolog LIN-35 (Cui & Han, 2007). Studies have suggested that these factors may mediate chromatin dynamics and posttranscriptional modifications, including histone tail deacetylation, methylation, acetylation and sumoylation, although for the most part, the associated mechanisms remain uncharacterized (Poulin et al., 2005; Fay & Yochem, 2007).

Studies indicate that SynMuv factors mediate cell fate decisions in a number of contexts, in addition specification of vulval cells. For instance, loss of individual

SynMuv genes induces ectopic expression of the distal tip cell reporter lag-2::GFP in gut and endodermal cells, indicating non-redundant transcriptional repression outside vulval tissues (Poulin et al., 2005; Cui et al., 2006). SynMuv genes also affect the specification of pharyngeal cell fate. The TRIM/RING SynMuv B protein TAM-1 physically associates with the pharynx selector factor PHA-4/FoxA by yeast two hybrid, and *tam-1* and NuRD components function synergistically with the pharyngeal selector gene *pha-4*, leading to a hypothesis suggesting that repression is mediated via direct interactions between transcription factors and NuRD components (Li et al., 2004; Kiefer et al., 2007).

Many SynMuv genes promote development of somatic cells by preventing expression of germline genes in the soma; SynMuv mutants (i.e., *mep-1*) misexpress germ line-specific P-granule proteins in somatic cells (Unhavaithaya et al., 2002; Wang et al., 2005; Petrella et al., 2011). However, these cells are not completely converted to germline like fates, as somatic genes remain correctly temporally and spatially expressed (Unhavaithaya et al., 2002). In addition, perturbation of several SynMuv B genes results in enhancement of RNAi and transgene desilencing, phenotypes often associated with the germ line (Lehner et al., 2006; Unhavaithaya et al., 2002; Wang et al., 2005; Petrella et al., 2011). Surprisingly, acquisition of germline characteristics does not appear detrimental to somatic development, as most SynMuv mutants grow to adulthood when cultured at low temperatures (Andersen et al., 2008). The ability of SynMuv mutants to develop appears to be temperature dependent, as culture at 26^{o}C induces high temperature arrest (HTA) and ectopic expression of germ line genes in the soma, while other SynMuv mutations (i.e., *mep-1* and *hpl-2*) are temperature sensitive at 25^{o}C (Petrella et al., 2011; Unhavaithaya et al., 2002; Schott et al., 2002).

Notably, loss of germ line regulators, including *mes-2*, *mes-3*, *mes-4*, *mes-6* and chromodomain containing *mrg-1* are able to suppress SynMuv induced somatic fates. Loss of these factors rescues larval arrest phenotypes in both SynMuv mutants and HTA, and suppresses the expression of germ line proteins in somatic cells (Unhavaithaya et al., 2002; Cui et al., 2006; Takasaki et al 2007; Wang et al., 2005; Petrella et al., 2011). These data indicate that the SynMuv factors antagonize MES repression of germ line genes. In addition, a number of chromatin factors, including MES-4, MRG-1, GFL-1 and ZK1127.3 function as SynMuv suppressors in that loss of these factors inhibits the formation of multiple vulval structures in SynMuv A/B mutants (Wang et al., 2005; Cui et al., 2006).

The repression of SynMuv factors is also key in the insulation of the germ line from somatic cell fate commitment (Shin & Mello, 2003). In the early germ line blastomere (P4), transcriptional repression is maintained by maternally donated PIE-1, which inhibits the NuRD complex thru direct repression of deacetylase activity (Unhavaithaya et al., 2002). Models suggest that during this period of germline specific transcriptional repression, the surrounding somatic cells are able to respond to differentiation factors. Stage specific patterns of chromatin organization are established within each cell lineage thru the concerted action of transcriptional activators and repressors, with MES factors promoting the germline like fate, and SynMuv genes promoting somatic fates. PIE-1 inhibition of MEP-1/LET-418/HDA-1 is thought to prevent chromatin remodeling and preserve MES dependent germline pluripotency during the early embryonic phase of somatic cell fate specification (Unhavaithaya et al., 2002). As the major role of MES factors is thought to involve repression of the X

chromosome, it has been proposed by some researchers that desilencing of genes on the X chromosome may contribute to the suppression of SynMuv phenotypes.

Silencing by RNA: the role of MES and SynMuvs

In *C.elegans*, dsRNA can induce posttranscriptional and transcriptional gene silencing (PTGS and TGS, targeting mRNA and nuclear pre-mRNAs respectively). These silencing mechanisms may have evolved for protection of the genome against viruses and transposable elements, as well as to regulate endogenous gene expression and/or the formation of heterochromatin. In this system, RNA-based mechanisms are required for RNAi (silencing of exogenous dsRNAs), germline silencing of transponsons, transgenes, and the phenomenon termed cosuppression, in which the endogenous gene locus becomes silenced by the presence of a repetitive array carrying the target sequence (Ketting et al., 1999; Tabara et al. 1999; Kelly et al. 1997; Dernburg et al. 2000; Ketting and Plasterk 2000). Numerous studies have demonstrated the existence of partially overlapping pathways controlling these mechanisms through the activity of *rde* (RNAi deficient), *mut* (mutator, involved in transposon silencing), and *cde* (cosuppression defective) genes (Ketting & Plasterk, 2000). The silencing of transposons and repetitive elements (including transgenes and transgene induced cosuppression) occur predominantly in the germ line, although these processes differ in their requirements for specific RNAi associated factors (Ketting and Plasterk, 2000). Intriguingly, many chromatin associated factors are involved in RNA mediated silencing pathways, including both RDE-1 dependent and independent silencing mechanisms.

Chromatin factors shown to play a positive role in RNAi include MES-4, GFL-1, and ZK1127.3; RNAi is less effective in these mutants (Dudley et al., 2002; Kim et al., 2005; Cui et al., 2006). MES-3 and MES-6 may also play a role in RNAi, although these data are less clear, and conflict with an earlier study (Dudley et al., 2002; Tabara et al., 1999). The concentration of dsRNA injected, as well as the degree to which these proteins are knocked out (i.e., null mutation vs. RNAi), suggests that the PRC2 complex may play some indirect role in RNAi silencing pathways (Dudley et al., 2002; Tabara et al., 1999). Alternatively, perhaps the MES/PRC2 complex plays a role in transcriptional gene silencing, and may be triggered in response to high doses of injected dsRNA but not low dose or feeding RNAi. However, *mes-2* was not found to effect RNAi mediated silencing in these assays, in contrast to *mes-3* and *mes-6* (Dudley et al., 2002). A number of chromatin associated factors, including MES-4, HPL-2, MRG-1, and subset of RNAi associated factors are also required for cosuppression, suggesting that cosuppression is also an RNA based silencing mechanism, perhaps similar to those mechanisms targeting repetitive elements (Robert et al., 2005). The same subset of RNAi factors required for cosuppression are required for transposon silencing; however, MRG-1 and MES genes do not appear to be essential for the silencing of transposons, suggesting that although the RNA based silencing mechanisms overlap, they are unlikely identical, perhaps requiring different factors depending on the targeted loci (Vastenhouw et al., 2002; Robert et al., 2005). While different from each other, cosuppression and transposon silencing mechanisms also differ from exogenous RNAi mechanisms, and are thus thought to occur at the transcriptional level (Grishok, 2005). Additional chromatin factors are involved in transcriptional gene silencing of transgenic arrays (reduced somatic expression, reduced

pre mRNA), including HPL-2, GFL-1, SET-2, and MRG-1 (Grishok et al., 2005); however, this screen did not identify the Mes factors. HP1 and PIWI proteins directly associate during repeat induced gene silencing, further supporting a role for RNA based chromatin regulation in transcriptional repression (Moazed, 2009). The fact that a number of genes antagonize RNAi silencing (i.e., *eri-1*, *rrf-3*), further suggests that RNA-based mechanisms of transcriptional silencing likely function during endogenous gene regulation.

SynMuv genes also play a role in RNAi, and like other functions, appear to oppose that of MES-4. RNAi is enhanced in the *lin-15B(n744)* mutation, suggesting that this protein negatively regulates RNAi (Wang et al., 2005). Other SynMuv factors shown to negatively regulate RNAi include LIN-35 (Rb), DPL-1 (DP), LIN-53, LIN-9, LIN-13 and HPL-2 (HP1); these genes are epistatic to some RNAi genes (i.e., *rde-1/4*, *mut-16*, *mut-7*), but not somatic RdRP *rrf-1* (Wang et al., 2005; Lehner et al., 2006). These SynMuv B genes also function synergistically with *eri-1* and *rrf-3*, genes involved in dsRNA synthesis and turnover (Wang et al., 2005). However, not all SynMuv genes function in the RNAi pathway (i.e., *lin-36* and *tam-1*), nor do all genes that enhance RNAi function as SynMuv factors (Lehner et al., 2006; Cui et al., 2007; Wang et al., 2005). Moreover, RNAi machinery is not required for the Muv phenotype, indicating that changes in RNAi efficacy and/or RNA mediated silencing are unlikely to be the underlying cause of vulval defects (Lehner et al., 2006; Cui et al., 2007; Wang et al., 2005). It has been postulated that enhanced RNAi in SynMuv mutants may be due to the conversion of somatic cells to a more germline like state; however, loss of the germ line gene *pgl-1* had no effect on RNAi sensitivity of SynMuv mutants, suggesting that

enhanced RNAi is not likely attributable to upregulation of germline expressed RNAi factors (Cui et al., 2006). This is also supported by the observation that some SynMuv mutations (i.e., *lin-35*) show enhancement of germ line RNAi, indicating that somatic misexpression of germline genes cannot solely account for enhanced RNAi (Lehner et al., 2007).

Inactivation of *rrf-3* or *eri-1* results in silencing of somatically expressed repetitive transgene arrays via an RNA dependent mechanism (Kim et al., 2005). A subset of SynMuv mutations also results in somatic transgene silencing, including *lin-35*, *lin-15B*, and *tam-1* (Hsieh et al., 1999; Lehner et al., 2007). Interestingly, loss of *tam-1* (Tandem Array expression Modifier) induces transgene silencing in the soma, but does not affect RNAi sensitivity, suggesting that these processes can be uncoupled at some level (Lehner et al., 2007). Somatic transgene desilencing in SynMuv B mutants (with the exception of *tam-1*) can be suppressed by depletion of RNAi (*dcr-1*) functionality, suggesting that increased RNAi in these mutants may induce the increased transgene silencing (Lehner et al., 2007). However, a previous study demonstrated that *tam-1* desilencing is Dicer dependent (Cui et al., 2006). *tam-1* dependent silencing of transgenes can be suppressed by depletion of *mes-4* or *mrg-1* (Cui et al., 2006). While *mes-4* is required for RNAi, it is currently unclear whether *mrg-1* plays a role in RNAi, adding an additional level of controversy (Cui et al., 2006). These data suggest that additional pathways function in somatic transgene silencing, perhaps by TGS. RT-PRC analysis demonstrated that *tam-1* dependent transgene silencing occurs at the pre mRNA stage, supporting this hypothesis (Cui et al., 2006). Moreover, the observed function of

MES-4 in RNAi may reflect a role in TGS, possibly by recruiting transcriptional silencing complexes to the targeted locus.

The mechanism of interaction relating to MES and SynMuv factors, and how these interactions mediate different processes including RNA based silencing and germ line/soma distinctions is unknown. These factors may function antagonistically to regulate the expression of a common set of target genes, and interact with these targets based on where they are expressed (i.e., soma vs. germ line). Alternatively, these factors might directly repress the expression of the other. A third model posits that MES-4 and SynMuv factors might compete for a common set of transcriptional or chromatin associated cofactors.

The enhanced RNAi phenotype associated with SynMuv mutation is postulated to be the result of derepression of germ line specific RNAi components in somatic tissue. It has been speculated that loss of SynMuv genes leads to inactivation of RNAi based heterochromatin formation pathways that would otherwise compete for the RNAi machinery. The release of RNAi machines from heterochromatin would then lead to the observed enhanced silencing of exogenous RNAi's.

siRNA silencing pathways appear to differ in the somatic and germ line tissues. This may be due to differential expression patterns, differences in sensitivity to RNA trigger molecules, or tissue specific expression of certain factors, such as the germ line specific RdRP EGO-1 (as opposed to the somatic specific RRF-1). EGO-1 is important for normal germ line development and chromosome segregation during meiosis, including deposition of H3K9me2 in a Dicer independent manner, suggesting again that RNAi pathways and/or machinery are utilized differently in different contexts (Maine et

al., 2005). In addition, some RNA based silencing pathway appear to function only in the germ line. For example, transposons are functional in the somatic tissue but silenced in the gonad (Vastenhouw & Plasterk, 2004). Cosuppression (also known as transgene induced phenocopy), is also primarily observed in the germ line and has only been noted for a single somatic gene (Dernburg et al., 2000). High copy transgenes are generally well expressed in the soma, but are silenced in the germ line (Kelly et al., 1997). These observations may be due in part to the germ line specific expression of repressive factors, including the Mes genes. An alternative, and nonmutually exclusive mechanism might involve somatic expression of adenosine deaminases (ADARs), which modify RNAs produced by transgenes and inhibit RNAi degradation (Knight and Bass 2002). Accordingly, transgenes are silenced in ADAR mutants in an *rde-1* dependent fashion (Knight and Bass 2002).

siRNAs and the formation of heterochromatin

An accumulating body of evidence indicates that small RNA molecules (20-30 nucleotides) may play a significant role in gene silencing by mediating the formation of heterochromatin, and this may reflect the underlying role of chromatin factors in the RNA mediated silencing described above. Noncoding RNAs that function as substrates for RNAi have been observed in a number of systems, and high throughput sequencing has demonstrated that *C.elegans* express several classes of endogenous small RNAs, including microRNAs, piRNAs, and endo siRNAs (Gent et al., 2010; Maine et al., 2010; Pavelec et al., 2009; Ambros et al., 2003; Ruby et al., 2006; Pak and Fire, 2007).

RNAi mediated formation of heterochromatin has been well studied in fission yeast, where small ncRNAs direct the formation of heterochromatin at repetitive regions, including the centromeres (Grewal, 2010). In this system, the RITS complex (for RNA induced transcriptional silencing) interacts with nascent RNAs, targeting RNA processing machinery (including RNA dependent RNA polymerases), and the H3K9 HMT Clr4 to target loci, specifically centromeric repeats (Grewal, 2010). Using the chromodomain of the RITS component Chp1, the complex binds methylated histones and induces a self-reinforcing feedback loop, facilitating the spread of silencing throughout the repeat region, an effect which is dependent on the activity of the RNA directed RNA polymerase (RdRp) RDP1, AGO1, and Dicer, core components of the RNAi machinery (Grewal, 2010). Loss of any of these components leads to reduced heterochromatic gene silencing and loss of H3K9me2/3 at centromeric repeats (Moazed, 2009). Studies in yeast also suggest that the RNAi machinery may directly interact with RNA PolII (Moazed, 2009).

The tethering of RNA processing machinery and the formation of heterochromatin appears to be conserved mechanism. In mammalian cells, introduction of siRNAs leads to the acquisition of repressive marks including H3K9 and H3K27 methylation at targeted promoters, as well as loss of histone acetylation, and recruitment of HP1 in an Argonaute dependent manner (Kim et al., 2006; Morris et al., 2004). ChIP experiments indicate that a similar mechanism may be used in *C.elegans*. In this system, the nuclear RNAi WAGO class Argonaunte protein NRDE-2 (nuclear RNAi defective) complexes with exogenous siRNAs, binds genomic target sites, and represses gene expression through H3K9me and inhibition of RNA PolII elongation (Guang et al., 2010;

Burkhart et al., 2011). siRNAs direct H3K9me to target loci in a NRDE dependent manner and induce PolII stalling; ChIP data show decreased H3K9me at siRNA targeted genomic regions in NRDE mutants, coincident with increased expression from the target locus (Burkhart et al., 2011). These data demonstrate that endogenous siRNAs are necessary and sufficient to induce changes in chromatin and transcriptional status.

However, the siRNAs in this study targets genes that were highly repetitive, poorly conserved, and perhaps nonfunctional (Burkhart et al., 2011). Therefore, it is currently unclear what role the various endogenous small RNAs play in chromatin regulation. These data do, however, demonstrate that endogenous RNAi-mediated changes in chromatin and histone modifications can be targeted to genes that are not embedded in constitutive heterochromatin as has been observed in yeast, providing us with new insight into mechanisms of gene silencing.

MES-4 & H3K36me

The H3K36me2/3 mark is present in all nuclei during embryogenesis, and is catalyzed by the HMTs MES-4 and MET-1 (Bender et al., 2006; Furuhashi et al., 2010; Andersen & Horvitz et al., 2007). Early embryos lacking MES-4 activity (M-Z-) also lack H3K36me2/3, indicating that these marks are MES-4 dependent (Bender et al., 2006). However, MET-1 becomes active at ~40 cell stage, possibly in conjunction with the upregulation of zygotic activity; accordingly, H3K36me2/3 is observed in *mes-4*(-) embryos after the 40 cell stage (Andersen & Horvitz et al., 2007; Bender et al., 2006; Reichsteiner et al., 2010). MET-1 activity appears to be restricted to the somatic tissue

until late stage embryogenesis, but does eventually contribute H3K36me to PGC chromatin in *mes-4* mutants (Furuhashi et al., 2010).

From late embryogenesis through adulthood, MES-4 mediated H3K36me2/3 is restricted to the germ line blastomere and PGCs, consistent with the observed localization pattern of MES-4 (Fong et al., 2002; Bender et al., 2006; Reichsteiner et al., 2010). In *met-1* (-) embryos, MES-4 mediated H3K36me2/3 is present throughout embryogenesis, and although the mark decreases over time in somatic cells, H3K36me2/3 is at wildtype levels in the PGCs (Furuhashi et al., 2010, Rechsteiner et al., 2010). H3K36me2/3 is absent throughout embryogenesis in *met-1; mes-4* double loss of function embryos, suggesting that MET-1 and MES-4 are the only embryonic HMTs catalyzing this mark (Furuhashi et al., 2010, Rechsteiner et al., 2010).

Most identified H3K36 HMTs, including MET-1, are recruited to genes through association with elongating RNA PolII, and are involved in preventing aberrant transcription initiation within the gene body (Lee & Shilatifar, 2007; Furuhashi et al., 2010; Rechtsteiner et al., 2010). In yeast, the RNA PolII CTD Serine 2 phosphorylation (a mark associated with transcriptional elongation), recruits the MET-1 homolog SET-2, which methylates nearby nucleosomes and recruits the Rpd3S complex (Lee & Shilatifar, 2007). The Rpd3S complex deacetylates nucleosomes within the coding regions leading to suppression of aberrant intragenic transcriptional initiation (Lee & Shilatifar, 2007). This mechanism appears to be well conserved in yeast and higher eukaryotes, and may also be used to distinguish actively transcribed sequences, inactive genes, and regulatory sequences (Lee & Shilatifar, 2007).

In contrast to most characterized H3K36me HMTs, MES-4 associates with target genes independently of RNA Pol II, as RNAi of the knockdown of the PolII large subunit AMA-1 does not result in loss of MES-4 mediated H3K36me2/3, whereas loss of *ama-1* dramatically decreases the level of MET-1 catalyzed H3K36me2/3 (Bender et al., 2006; Rechtsteiner et al., 2010; Furuhashi et al., 2010). In *mes-4* mutant germ lines (M+Z-), H3K36me2 is absent in the mitotic distal region through pachytene. Experiments using *mes-4* and *met-1* mutants, combined with RNAi knockdown of *ama-1* have demonstrated the germ line contains MES-4 dependent, transcription independent H3K36me, as well as H3K36me that is dependent MET-1 and ongoing transcription. While the majority of MES-4 mediated H3K36me2/3 in embryos and the germ line appear to be independent of transcription, a PolII dependent function of MES-4 has not been ruled out.

These observations, coupled with the finding that MES-4 is incapable of *de novo* methylation of H3K36, led to a model proposing that MES-4 functions as a maintenance methyltransferase (Furuhashi et al., 2010; Rechtsteiner et al., 2010). As *met-1* mutants are homozygous viable and fertile despite loss of ~90% of H3K36me3 in mixed stage embryos by western blot, it seems that *mes-4* is 1) extremely efficient at maintaining the remaining H3K36me, and/or 2) H3K36me is not essential in somatic tissues or 3) a different histone mark functions redundantly to mark transcribed genes, and/or 4) MES-4 functions in some essential capacity in addition to H3K36 maintenance, as suggested by the maternal effect sterile phenotype associated with *mes-4* but not *met-1*(Andersen & Horvitz 2007; Furuhashi et al., 2010).

Chromatin immunoprecipitation based analyses, including ChIP-chip and ChIP-Seq performed on embryo lysates provide further insight into MES-4/H3K36me localization and function. First, MES-4 and H3K36me2/3 ChIP signals on all genes are highly correlated (Reichsteiner et al., 2010). Second, MES-4 binding is associated with the coding region of genes, consistent with previously studies implicating H3K36me in splicing (Rechsteiner et al., 2010; Lee & Shilatifar, 2007). Third, 1 in 5 genes is bound by MES-4, and many of these targets are also bound by RNA PolII (Rechsteiner et al., 2010). Fourth, a higher level of MES-4 binding correlates with higher levels of transcription, although PolII peaks at the 3' end of a transcript and MES-4 peaks at the 5' end, lending further support to the notion that MES-4 function is uncoupled from PolII and transcriptional activity (Rechsteiner et al., 2010).

The most striking finding of these embryonic ChIP-based studies is that MES-4 and MES-4 dependent H3K36me2/3 mark genes that were expressed in the maternal germ line, but are not expressed in embryos and are thus not associated with RNA PolII (Reichsteiner et al., 2010; Furuhashi et al., 2010). ChIP-Seq data collected from *met-1* mutant embryos demonstrate that H3K36me3 is primarily associated with this same cohort of genes, further implicating dependence on MES-4 (Furuhashi et al., 2010). Moreover, H3K36me3 is absent from germ line specific genes in *mes-4* mutants, while embryo and soma specific genes are still marked by H3K36me in this context, presumably due to transcription-dependent MET-1 activity (Rechsteiner et al., 2010). The finding that MET-1/transcription dependent H3K36me3 is 3' enriched, and MES-4/transcription-independent H3K36me is more 5' enriched (and in many cases may still be associated with MES-4) may provide a method for downstream effector proteins to

differentiate between these marks, allowing for expression in the soma or germ line, respectively. In yeast, H3K36me2 and H3K36me3 marks have different functions, with H3K36me3 correlating with transcriptional frequency and H3K36me2 linked to transcriptional on/off state (Shilatifard, 2006). However, it is currently unclear whether these marks represent different functions in *C.elegans*, and whether they are differentially regulated by MET-1 and MES-4.

MES-4 is also required for the PIE-1 independent phase of transcriptional repression in the PGCs (Furuhashi et al., 2010). In wildtype animals, RNA PolII becomes transiently activated in Z2/Z3 following the division of the P4 cell (Strome, 2005). This activation appears to be regulated in a unique manner, as antibody stains indicate that PolII hyper phosphorylation in these cells is independent of *cdk-9*/P-TEFb (Furuhashi et al., 2010). Intriguingly, PolII activity remains high in the PCGs in *mes-4* mutant embryos, but is unaffected by loss of *mes-2* (Furuhashi et al., 2010). In *mes-4*(-) embryos, H3K4me2 (a mark of transcriptional activity), is initially erased following the birth of the PCGs (as in wildtype embryos), but are significantly increased in late stage embryos relative to controls (Furuhashi et al., 2010). These data demonstrate that MES-4 functions to repress transcriptional activity in the PGCs. It is likely that this inhibition occurs at the level of PolII activity, as CTD phosphorylation state appears to be the primary defect, with H3K4me increasing as an effect of increase PolII activity. The polymerase transcriptional machinery remains excluded from the X chromosome in the PGCs, which may be consistent with *mes-2* independence (Furuhashi et al., 2010). Interestingly, the NuRD complex does not appear to function in the chromatin remodeling and germline repression of Z2/Z3 following degradation of PIE-1, suggesting

that the role of MES-4 in these cells may be independent of SynMuv function, or alternatively, interacts with a SynMuv-like complex other than NuRD (Schaner et al., 2003).

As maternal MES-4 and marks associated with gene expression in the germ line are initially present in all cells of the early embryo, expression of germline genes is likely antagonized in somatic blastomeres. The SynMuv class of transcriptional repressors might be ideal candidates to block or erase marks associated with MES-4 mediated activation, as SynMuvs and MES-4 have opposing functions in a number of biological contexts. The marking of germ line expressed genes by MES-4/H3K36me may be required for the observed upregulation of germ line expressed genes in the soma of SynMuv mutants, where global transcriptional repression mechanisms are defective, with H3K36me2/3 inducing default expression when somatic repressors are absent (as in the germ line) or defective (in mutants) (Furuhashi et al., 2010).

The loss of function phenotypes associated with *mes-4* are similar to those observed in *mes-2/3/6* mutants, including maternal effect sterility, germ cell necrosis, sensitivity to X chromosome dosage, and silencing of transgenic arrays in the germ line (Capowski et al., 1991; Garvin et al., 1998; Kelly et al., 1998). However, many phenotypes associated with *mes-4* are stronger than those associated with other *mes* genes, including reduction in germ cell nuclei and suppression of SynMuv defects, including suppression of high temperature arrest (Capowski et al., 1991; Cui et al., 2006; Peteralla et al., 2011). Other phenotypes appear to be *mes-4* specific. For instance, *mes-4*, but not *mes-2* is not required for the repression of PolII activity in embryonic PCGs, further distinguishing the MES/PRC2 complex from MES-4 (Furuhashi et al., 2010). MES-4,

but not the other *mes* genes, also appears to play a more direct role in RNAi pathways (Cui et al., 2006; Wang et al., 2005; Dudley et al., 2002; Kim et al., 2005; Tabara et al., 1999). *mes-4*, but not *mes-2/3/6,* function in the cosuppression pathway and suppress the multi-vulva phenotype associated with SynMuv mutants (Cui et al., 2006; Robert et al., 2005). Based on these data, it has also been suggested that the suppression phenotypes associated with *mes-2/mes-3/mes-6* may reflect the role of MES/PRC2 in localizing MES-4 activity.

To summarize, MES-4 is an autosome enriched HMT essential for repression of the X chromosome, marking germ line specific genes with H3K36me, maintenance of PolII/MET-1 dependent H3K36me3, and inhibition of PolII activity in the PGCs. Studies have indicated that HMT activity is essential for the maternal function of MES-4; however, whether these newly defined functions are linked and/or dependent on HMT activity remains to be addressed.

Chromatin compaction

Previous work in the Mango lab has demonstrated a role for MES-2/PRC2 in the regulation of chromatin compaction, specifically during the transition from a plastic to a cell fate committed stage, which occurs during the 2E to 8E stages of development. At the 2E stage, when cells of the embryo are plastic and able to adopt a number of cell fates, enrichment of the open chromatin configuration is observed using the Nuclear Spot assay (Yuzyuk et al., 2009). During the 4E and 8E stages, when wildtype cells begin to commit to specific fates, the proportion of "open" arrays decreased, with a concomitant increase in compact, nuclear envelope associated morphology (Yuzyuk et al., 2009). These

compact arrays are likely heterochromatic, as immunofluorescence analyses have demonstrated enrichment of H3K27me3 and depletion of activated RNA PolII when compared with the open (floret) counterparts (Yuzyuk et al., 2009).

In *mes-2* mutants, a large proportion of arrays remained in the euchromatic floret configuration even in 4E and 8E stages, while a reduced number of heterochromatic morphologies were observed (Yuzyuk et al., 2009). Based on these data, we hypothesize that MES-2/PRC2 functions to compact chromatin during the transition from pluripotency to differentiation, likely by direct targeting of nucleosomes and histone tails modification (Yuzyuk et al., 2009), a finding consistent with studies of PRC2 function in other species (Erwin and Lee, 2008; Zaratiegui et al., 2007; Margueron et al., 2008). Although indirect means of chromatin compaction regulation have not been ruled out, an intriguing hypothesis is that global chromatin reorganization driven by PRC2 contributes to the termination of plasticity.

The timing of pluripotency termination is delayed in the absence of PRC2 activity, as blastomeres "challenged" by overexpression of cell fate selector genes are capable of undergoing changes in fate at the 8E stage (Yuzyuk et al., 2009). This finding was somewhat unexpected. Based on the current understanding of PRC2 function, one might predict that loss of *mes-2* would lead to an early onset of differentiation. Moreover, loss of MES-2/PRC2 activity results in gene expression changes, including failure to upregulate a cohort of 8E (differentiation) genes as well as precocious/sustained activation of some developmental regulators at the 2E stage. Embryos eventually overcome the sustained expression, and develop normally, indicating the presence of redundant pathway(s) leading to cell fate commitment, a hypothesis supported by the

finding that target genes of upregulated (derepressed) developmental regulators are expressed in normally in late stage embryos. This analysis also demonstrated that developmental regulators (i.e., selector genes) are not key terminators of plasticity, and that cell fate restriction can be uncoupled from cell fate specification, a feature which distinguishes developing embryos from ESC lines, in which pluripotency is associated with arrested development and a static expression landscape (Rossant, 2008).

Multiple lines of evidence suggest that the role of MES/PRC2 in termination of developmental plasticity is carried out in the soma, rather than the germ line. Although the previously ascribed functions of MES/PRC2 deal with silencing of the X chromosomes in the adult germ line, the altered expression profiles of *mes-2* mutants did not show a bias for X linkage, in contrast to expression profiles of *mes-4* mutants (Yuzyuk et al., 2009; Bender et al., 2006). The finding that loss of *mes-2* activity has little effect on early embryonic gene expression (<4E), including activation of zygotic genes, also suggests a specific temporal role after the 4E stage (Yuzyuk et al., 2009). Instead, loss of *mes-2* led to inappropriate activation of zygotic developmental regulators, as has been observed in PRC2 in other systems (Yuzyuk et al., 2009; Schuettengruber et al., 2007; Niwa, 2007). Moreover, the altered expression profile in *mes-2* mutants was not dependent on the germ line, as similar changes in gene expression were observed in mutants lacking a germ line (Yuzyuk et al., 2009). Previous analysis identified subtle phenotypes in PRC2 mutants during postembryonic development and associated with regulation of *Hox* genes (Ross & Zarkower, 2003; Zhang et al., 2003). Data from the Mango lab, however, clearly demonstrate a role for PRC2 in transcriptional regulation of the soma during embryonic development.

Loss of MES-2/PRC2 function leads to prolonged plasticity in the early embryo, possibly resulting from a failure to compact the permissive chromatin configuration and/or downregulate 2E expressed factors. Despite inappropriate gene expression, downstream targets of these developmental regulators are unaffected, and worms are viable, suggesting the presence of additional levels of control.

C.elegans embryos appear to function differently from other species in the degree to which transcription factors direct pluripotency programs during development. Of the five classes of TFs know to contribute to pluripotency in mammals (Oct4/Pou/*unc-6*, *unc-86*, *ceh-18*, Sox2, Nanog/Klf/Kruppel-like/*nos-1*/*nos-2* and c-Myc/*mml-1*), the known *C.elegans* homologs are not clearly linked to developmental plasticity. They are either not expressed during this early transition period (*unc-6, unc-86, ceh-18*), have no discernable embryonic phenotype (*mml-1*), or function in some alternative capacity (*mep-1*) (Unhavaithaya et al., 2002; Pickett et al., 2007; Baugh et al., 2003; Yuzyuk et al., 2009). These data indicate that the transition from pluripotency to cell fate commitment in *C.elegans* may rely on mechanisms distinct from those characterized in other species.

SET-2: A previously identified PcG interacting factor

SET-2 is related to yeast Set1 and mammalian SET1/MLL proteins (Xu et al., 2001). Mixed lineage leukemia (MLL), its *Drosophila* homolog (Trithorax), and the yeast SET1 protein are each found within multisubunit COMPASS (Complex of proteins associated with Set1)-like complexes involved in activation of gene expression through methylation of H3K4 (Eissenberg & Shilatifard, 2010). Depletion of SET-2 was found to enhance *mes-3* and *mes-4*, leading to enhanced sterility in the F_1 (M+Z-) generation, as

well as increased desilencing of transgenic arrays (Xu et al., 2001). The finding that *set-2* enhances *mes-3* and *mes-4* alleles, but not *mes-2* and *mes-6*, might be attributable to the stronger phenotypes associated with loss of *mes-3* and *mes-4*, which the authors suggested might relate to perduance of the maternal endowment (Xu et al., 2001). An alternative interpretation suggests that MES-3 may have functions in addition to those of MES/PRC; likewise, MES-4 has been linked to a number of functions in addition to X-chromosome silencing. SET-2/COMPASS may play a role in mediating these functions.

Similar to the MES genes, SET-2 is germline enriched and localized to nuclei in the germ line and early embryos (Xu et al. 2001). In the germ line, SET-2 localization is highly dynamic, with enrichment in the distal mitotic zone, reduced in the transition zone and early pachytene, followed by dramatic upregulation in midpachytene, reduction in oocytes, increase in early embryos, and slowing fading in many somatic lineages during embryonic development while remaining enriched in the germline progenitor cells (Xu et al., 2001). This localization pattern mimics what has been observed for MES factors, lending support to the hypothesis of a common pathway. Like MES-4, the dynamic localization of SET-2 does not require other MES factors, nor do the MES proteins require SET-2 for localization or HMT activity (Xu et al., 2001). Therefore, the interaction between SET-2 and the MES factors is currently unclear. One possibility is that SET-2 and MES serve redundant functions in setting up the maternal germline chromatin. Alternatively, SET-2/COMPASS mediated marks may contribute to activation of X associated repressors.

The SET-2 protein, in addition to the methyltransferase domain, contains both a proline rich protein-protein interaction domain, and an RRM domain that may bind RNA

(Xu et al., 2001). SET-2 lacks the cysteine-rich region upstream of the SET domain, and may not possess HMT activity, but instead may mediate protein-protein interactions via the SET domain, similar to the Trithorax (Xu et al., 2001). However, a recent study demonstrated that H34me2 and H3K4me3 are reduced 70-80% in *set-2(-)* embryos and young adults, demonstrating that while functional SET-2 is essential for H3K4me, an additional H3K4me HMT is also active during development (Xiao et al., 2011). By western blot, SET-2 is responsible for more than 90% of germline H3K4me2/3, suggesting that SET-2 may function primarily in this tissue (Xiao et al., 2011). With the exception of SET-2, no additional proteins have been linked to the Mes/PcG pathway, although genetic data, the subtle somatic phenotypes associated with loss of MES function, and biochemical evidence suggest that additional factors may function in this pathway, if not in the PRC2 complex itself.

Mes factors in the soma and the regulation of *Hox* genes

The evolutionarily conserved PcG proteins are best known for their role in maintaining the repression of homeotic genes during embryonic development. Antagonized by the Trithorax factors, which maintain active gene expression within specific domains, PcGs allow for refinement of positional expression along the anterior/posterior body axis. *Mes* mutant M+Z- animals have a reduced number of progeny relative to wildtype controls, suggesting that MES proteins also play a zygotic role (Capowski et al., 1991). However, there is no increased embryonic death, indicating that the gene products are not required for embryonic viability (Capowski et al., 1991; Paulsen et al., 1995). Based on these data, MES factors may have some nonessential role

in the soma (in addition to chromatin compaction during cell fate specification), which would be consistent with Drosophila studies demonstrating a role in *Hox* gene regulation.

As homeotic repression is a feature of PcG in Drosophila, *C.elegans* researchers have been interested in the role of MES PRC2 factors in the regulation of *Hox* genes. In addition to the developing and adult germ line, the PRC2 complex is also present at low levels in at least some somatic tissues, where Hox regulation would be expected to take place. Tissue specific expression analysis using various mutants which produce either no germ line, and masculinized or feminized germ lines indicate that expression of *mes-3* is not restricted to the germ line tissues (Paulsen et al., 1995). In addition, antibody staining experiments have demonstrated that the presence of MES-2 and MES-6 in intestinal nuclei throughout development (Korf et al., 1998; Holderman et al; 1998). Intriguingly, ectopic induction of germ line markers following high temperature arrest (Petrella et al., 2011) and loss of *mep-1* (Unhavaithaya et al., 2002) are strongest in the intestinal tissue, perhaps indicating that the higher levels of MES are required to maintain repression in these cells.

PRC2 components MES-2/3/6 appear to function upstream of the *Hox* genes *mab-5* and *egl-5* during ray V differentiation in the male tail, as loss of *mes* activity restores normal ray development and mating ability in males mutant for *Hox* activators in a Wnt independent manner (Ross & Zarkower, 2003). As Wnt signaling appears to be one of the main regulators of *Hox* expression, this indicates that MES factors may function in a separate pathway. In addition, reporter assay based analyses demonstrated a small but significant change in the patterning of other Hox genes (*lin-39*, midbody; *mab-5*, neuronal lineage), suggesting that the MES genes are negative regulators of *Hox* genes

during larval development, an effect that is not restricted to males, or development of the tail rays (Ross & Zarkower, 2003). While these effects are fairly subtle, they do support MES factors playing some role in somatic development, although whether this effect is direct has not been fully established. Whether this repression might be set up in the embryo and persisting until larval stages has also not been addressed.

The finding that loss of H3K27me3 is tolerated during embryonic development is surprising, and raises questions about the role of this mark in the somatic tissues. Loss of *mes2/3/6* results in low penetrance homeotic transformations (in which the body structures are duplicated or lost), including defects in migration of specific neurons, expansion of *Hox* gene expression domains, and mislocalization of sensory rays in the male tail, as well as sexual transformations in certain genetic backgrounds (Korf et al., 1998; Holdeman et al., 1998; Ross and Zarkower, 2003; Garvin et al., 1998).

The PRC1 complex in *C.elegans*

Work in other systems has demonstated the existence of the PRC1 complex, which is thought to recognize H3K27me3 and ubiquitinate H2A lysine 119, resulting in chromatin compaction and transcriptional silencing. However, whether this complex exists in *C.elegans* has been a subject of debate. Two complexes, the *sop-2/sor-1/sor-3* complex, and the *mig-32/spat-3a* complex have been suggested to function in this capacity.

Loss of SOP-2 complex activity is associated with shifts in male sensory ray position, number, and identity, abnormal neuroblast migration and expansion of homeotic expression domains, as well as *hox* dependent precocious differentiation and conversion

of alae fated seam cells into male tail specific rays (Zhang et al., 2003; Zhang et al., 2006; Cai et al., 2008; Yang et al., 2007). SOP-2 is a SAM-domain RNA binding protein that forms distinct nuclear bodies, a feature reminiscent of PRC1 components in other organisms (Zhang et al., 2006). While *sop-2* phenotypes resemble the *mes*/PRC2 phenotypes with respect to subtle homeotic transformation, the data supporting the SOP-2 complex functions as PRC1 are weak. First, mutations of proposed complex members have variable phenotypes indicative of at least partially independent functions. Loss of both *sop-2* and *sor-1* is synergistic, suggesting that these components may not function as a complex *in vivo*, a finding supported by immunofluorescence data demonstrating differential localization patterns (Zhang et al., 2006). These mutants also display pleiotropic defects (vulval, neuronal, gonadal) that are not attributable to *Hox* misregulation (Zhang et al., 2009). Based on these findings, it has been suggested that the misregulation of *Hox* expression is an indirect effect.

Genetic interactions between *sop-2* and the MES complex are also unclear. Synergy was not observed between *sop-2* and the *mes-2/3/6* with respect to the timing and extent of ectopic *Hox* gene expression, but synthetic larval lethality not related to *Hox* misexpression was observed (Zhang et al., 2009). The authors of this study did not examine whether *sop-2* and *sor-1* might function in one of the other pathways contributing to homeotic patterning (i.e., *Pry/Wnt*). As the homeotic defects associated with loss of MES function as so subtle compared to *sop-2* and *sor-1*, it seems unlikely that PRC2 function is a prequisite of *Hox* gene repression in *C.elegans*, provided that the SOP/SOR complex functions in this capacity (i.e., PRC1 like) at all. Moreover, it has not been shown that *sop-2* or *sor-1* bind H3K27me2/3, a mark that is recognized by PRC1 in

other species. Whether *sop-2* and *sor-1* are associated with ubiquitin ligase activity, the mechanism by which PRC1 is thought to mediate its repressive effects, has not been shown. Lastly, *sop-2* and *sor-1* homologs are not found even in *C.elegans* closest relative, *C.briggsae* (Zhang et al., 2009), indicating that these factors do not represent a conserved complex within the nematode phylogeny.

More recently, the MIG-32 and SPAT-3a have been proposed to represent the PRC1 complex in *C.elegans* (Karakuzu et al., 2009). These genes encode homologs of the PRC1 components BMI-1 (Ring domain) and Ring1B respectively. Consistent with these genes functioning in a complex similar to PRC1, loss of either *mig-32* or *spat-3a* leads to a marked reduction (*spat-3a*) or complete loss (*mig-2*) of H2A ubiquitylation by western blot of L1 lysates. However, localization of *mig-2* to nuclei is not *mes* dependent, and whether H2Aub levels decrease in a *mes* dependent manner has not been examined.

It was demonstrated by epistasis analysis that *mig-32* and *mes* genes function in a common pathway to control patterning of the male tail (Karakuzu et al., 2009). MIG-32 and SPAT-3a also play a role in neuronal migration and repression of vulval fates, similar to what has been observed in *mes* mutants (Ross & Zarkower, 2004; Cui et al., 2006; Karakuzu et al., 2009). Reporter analysis also suggest that misexpression of *Hox* genes may be the driving force behind these phenotypes (Karakuzu et al., 2009). Based on these findings, it has been suggested that MIG-32 and SPAT-3a represent the *C.elegans* PRC1 complex, however, both mutants are fertile in spite of broad germline expression, in contrast to the *mes* counterparts, indicating that ubiquitination of H2A by PRC1 is not essential for the germ line function of MES/PRC2. However, possible redundancy of H2A ubiquitinases in the germ line has not been fully examined.

Whether *C.elegans* has a complex similar to PRC1 remains unclear. The phenotypes associated with the genes proposed to function in this capacity do not resemble predictions based on PRC1 in other organisms, and it is unlikely, based on localization patterns, that MIG-32/SPAT-3a and SOR-2/SOP-1 function in a common complex, despite a similarity of homeotic phenotypes. Perhaps, as the homology data might suggest, *C.elegans* has evolved mechanisms of PRC2 repression that do not rely on PRC1 function. Alternatively, these complexes might function in Hox gene regulation with the MES/PRC2 complex, and the likely redundancy of additional pathways (i.e., Wnt) has made interpretation difficult. As the majority of cells in these various mutants are specified normally, it is unlikely that these factors are essential for the specification of cell fate, but instead may play a role in refinement of patterning following specification.

CHAPTER II

IDENTIFICATION OF NOVEL REGULATORS OF TRANSCRIPTIONAL REPRESSION AND ANALYSIS OF FUNCTION IN THE MES PATHWAY

Hypothesis and screen rationale

Loss of key silencing mechanisms induce surprisingly subtle defects with respect to somatic tissues and viability, indicating that additional factors may function in gene regulation and silencing pathways. Studies also suggest that additional regulators of heterochromatin formation remain to be identified. One example comes from *Drosophila*, where a recent genomewide ChIP-Seq analysis identified three types of heterochromatin, including areas silenced primarily by Polycomb and areas silenced by HP1 and interacting proteins (Filion et al., 2010). The third and most prevelent type of heterochromatin (occupying ~50% of the probed genome) is not occupied by marks or factors previously recognized as functioning in heterochromatin (Filion et al., 2010). These areas are not constitutively silenced, however, as genes expressed in various tissues are present, and transposons inserted into these areas undergo active repression (Filion et al., 2010). Analysis of these domains, as well as screens aimed at the

discovery of heterochromatin regulating factors, will provide a greater understanding of the regulation of gene expression throughout development.

There are several hints that additional factors may be involved in gene silencing and heterochromatin formation in *C.elegans*. One might predict that loss of gene repression and mechanisms of chromatin compaction might have fatal consequences, as loss of these regulators in other organisms result in embryonic lethality or homeotic transformation (Kwon & Workman, 2011; Surface et al., 2011). However, loss of any of the three major mechanisms of transcriptional repression (Polycomb, H3K9me, and HP1, depicted in Figure 4) have strikingly mild affects with respect to the soma. *mes-2* mutants, for instance, are able to produce progeny, and in these animals, differentiation is delayed, but not completely blocked, suggesting that additional mechanisms also regulate cell fate transitions (Capowski et al., 1991; Yuzyuk et al., 2009). Animals lacking MET-2 H3K9me2 HMT activity are viable and fertile for ~20 generations before germ line defects become evident (Andersen & Horvitz, 2007). Animals lacking HP1 activity are fertile, although progeny arrest at high temperature (Schott et al., 2006).

We are particularly interested in the identification of additional factors at work in the Polycomb/Mes silencing pathway. The goal of this screen is to identify chromatin associated proteins that interact with MES factors functioning during the transition from a state of cellular plasticity to fate committed cell types with fixed lineages. As the *C.elegans* genomes appears to lack, by sequence homology, a number of Polycomb factors, as well most regulatory subunits identified in flies and mammals, a directed screen should allow us to identify novel components and add to the growing body of

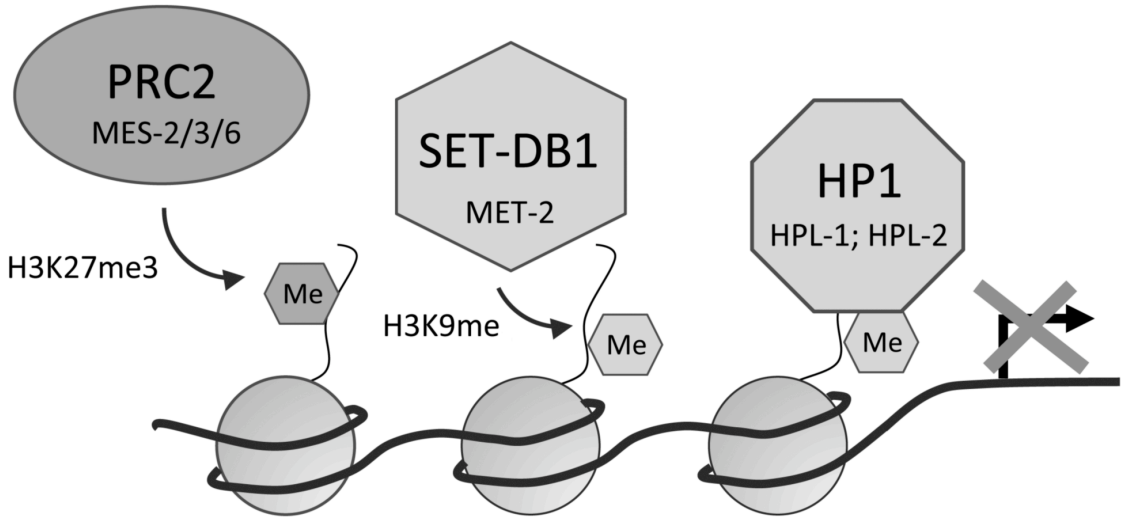


Figure 4. Key mechanisms of transcriptional silencing. In *C.elegans*, the PRC2 complex is composed of MES-2, MES-3 and MES-6, and catalyzes the repressive mark H3K27me3. The SET-DB1 ortholog MET-2 mediates H3K9me2/3, a silencing mark that in other systems is bound by heterochromatin protein 1 (HP1). In *C.elegans*, HP1 is encoded by the partially redundant HPL-1 and HLP-2.

research demonstrating the roles of Polycomb mediated silencing throughout development. *C.elegans* Polycomb factors may be sufficiently divergent from *Drosophila* and mammals, making recognition of PcG orthologs by amino acid sequence difficult, yet it is possible that proteins functioning in these complexes are functionally conserved. In addition, *C.elegans* might regulate Polycomb by mechanisms less likely to be completely unique than to shed light on the complex interplay of silencing mechansims we are only beginning to understand.

This type of analysis might also allow for identification of factors that may be partially redundant and allow for the subtle somatic effects of MES mutation. A precedent for this comes from mouse ES cells, where at least two enzymes are capable of catalyzing H3K27 methyl marks (Ezh1, Ezh2), both of which can associate with the core

PRC2 complex (Shen et al., 2008). This type of redundancy might also confer target specificity. A similar redundancy may be observed in *C.elegans*, where H3K27me2 and H3K9me3 are present in cells lacking expression of MES-2, although it is possible that this mark is residual and/or due to modulation of histone demethylases (Bender et al., 2004).

In addition, biochemical data has not ruled out the presence of additional factors in the PRC2 complex, although it seems unlikely. More probable are factors regulating the activity, recruitment to target loci, binding specificity, and/or mediation of downstream effects (i.e., chromatin compaction). One interesting hypothesis suggested by the literature is the possibility that PRC2 or MES-4 function in conjunction with additional complexes. These complexes might allow for the removal or addition of adjacent histone marks, and/or direct subsequent remodeling activity.

It is currently unknown whether *C.elegans* possess a PRC1-like complex, or whether an alternative complex exists. As independent functions of the PRC complexes have been observed in multiple species, the field would benefit from the discovery of additional proteins capable of reading the H3K27 methyl mark. It is also unclear how, after reading this mark, it is interpreted to generate heterochromatin formation. Possible models might include recruitment of additional factors which block the recruitment of transcription machinery, inhibit of PolII elongation, recruit of remodeling machinery to compact chromatin, remove machinery which keeping nucleosomes in an open configuration, and/or removal of activating histone marks such as acetylation and H3K4me2/3.

C.elegans provides an excellent system in which to study histone PTMs and the role in heterochromatin formation. As this system lacks DNA methylation at cysteine residues, it is likely that a subset of PTMs, histone variants, and effector proteins underlie all requisite chromatin regulatory signals, preservation of germ line identity, and heterochromatin formation during the transition from cellular plasticity to cell fate commitment, all of which are likely necessary for implementation of gene expression programs. It is also possible that the study of these worm genes may shed light of the roles of mammalian counterparts in development and disease.

This work describes an RNAi based candidate screen designed to identify mediators of transcriptional silencing in the nematode *C.elegans.* Findings of this study provide a unique opportunity to more fully characterize the Polycomb pathway in *C.elegans*, and may reveal novel mechanisms of heterochromatin formation functioning in the nematode as well as other organisms.

Methods: Sublibrary and primary screen

In order to identify additional factors functioning in transcriptional silencing, we designed a feeding RNAi based candidate screen to identify genetic enhancers of the maternal effect sterile *mes-3*, a Polycomb component required for the majority of H3K27me2/3 and H3K9me3 in the germ line. Genetic enhancers were also identified for *met-2*, a Su(Var)-93/SETDB1 ortholog required for H3K9me2, which when mutated, is superficially homozygous viable. It was possible to identify of both enhancers and suppressors of the partially redundant *hpl-1; hpl-2* HP1 mutant, as these alleles induce a larval arrest phenotype. The candidate library was composed of 738 RNAi clones

targeting 650 genes localized to the nucleus or involved in RNAi mechanisms by gene ontology (GO) terms.

Sublibrary construction

The RNAi sublibrary used in this screen was generated using a bioinformatics approach, using the GeneOntology database (http://amigo.geneontology.org). RNAi feeding clones were selected based on the GO term "Nuclear," where chromatin regulatory factors are likely to localize. As individual researchers annotate genes, classification terms are widely variable, and gens can be annotated by "molecular function" and "biological process" as well as "cellular component." In order to cull all genes of interest, genes associated with a range of GO terms were compiled, including, but not limited to: nucleus (1263), nuclear (323), nuclear part (211), nuclear lamin (2), nucleolus (7), nucleosome (27), nuclear envelope (37), transcription (509), transcription regulator activity (220), transcription factor binding (39), nucleic acid binding transcription factor activity (548), DNA (645), DNA binding (138), nucleic acid binding (1301), chromatin (131 genes), chromatin binding (25), chromatin modification (34), chromatin organization (140), chromatin assembly/disassembly (108), chromosome (129), nuclear chromosome (53), histone (243), nuclear lumen (153), DNA conformational change (102), gene silencing (146). As RNAi machinery is increasingly implicated in gene silencing (see introduction), the GO term RNA interference was also included (126 genes). However, as some genes are not annotated by GO terms, a proportion of genes were missed, although we also added some genes based on the presence of specific

domains (i.e., SET). The sublibrary gene list was considered saturated when a random group of known nuclear factors were listed by GO term analysis.

The FAS Center for Systems Biology Scriptome was employed to remove repeated genes, which was very common as genes are annotated with multiple GO terms. The sublibrary gene list was then narrowed by the removal of known transcription factors using Scriptome software to compare to the Walout lab transcription factor database (Reece-Hoyes et al., 2005). The sublibrary list was then annotated with "short descriptions" using the WormMart database export tool. Obvious DNA and RNA polymerases were removed from the list manually. By removing these characterized transcriptional elements, we hope to simplify the analysis and rule out potential false positives, increasingly the likelihood of identifying factors not previously associated involved in transcriptional regulation.

We are interested in genes involved in heterochromatin formation, as well as genes required during the transition from pluripotency to cell fate commitment. This transition occurs during the 2E-8E stages of embryonic development, and is accompanied by dramatic reorganization of nuclear architecture, changes linked by the function of MES/PcG function (Yuzyuk et al., 2009). Based on these findings, we limited our screen to genes that are expressed during the 2E-8E window. Genes not expressed at any point during this period by microarray analysis were excluded. Using these criteria, we identified 816 candidate genes.

RNAi clone assignment for individual genes was accomplished using the "Get locations" Perl script written in collaboration with Amir Karger (Harvard University FAS). This script was run against both commercially available genomewide RNAi

library databases (Ahringer and Vidal RNAi libraries), and multiple clones per gene were not excluded. As these genomewide libraries have ~90% gene coverage, only 738 clones out of 816 were available for screening purposes.

The sublibrary gene list was also annotated with previously identified RNAi phenotypes, specifically noting sterility, lethality and embryonic, larval, or adult arrest. Although this represents a large proportion of the candidate genes, these were included in the library to serve as internal positive controls and to test *hpl-1; hpl-2* suppression. Additional controls included empty vector (L4440) and *mes-2* RNAi. *mes-2*, rather than the stronger *mes-4,* was chosen for a control to ensure that a more subtle suppression could be reliable detected.

Picking RNAi clones

RNAi targeting clones were organized according to position in the parent plate (i.e., the 52 AhRNAi plates arrayed in 384 well format) and cherry picked into 150 ul of LB media + antibiotics (12.5 ug/ml tetracycline and 50 ug/ml carbenicillin) using autoclaved toothpicks into a 96 well format sublibrary (round bottom plates, Cell Star #650180). All pipetting was performed using autoclaved tips. All surfaces were sterilized with 70% EtOH and bleach. Gloves were worm at all times. Parent plates were removed from the -80^{o}C freezer two at a time, and kept on dry ice during the cherry picking process, before being resealed with aluminum and returned to -80. These "first pass plates" were grown overnight in a humidified shaker at 37^{o}C (rpm <200 to avoid spillage/contamination). In a subset of wells, bacteria failed to grow; these RNAi clones were repicked from the Mango Lab libraries and/or the Hunter lab Ahringer collection,

grown overnight and used to supplement specific wells. A sterilized hedgehog was then used to replate the first pass plates; these second pass plates were grown 8 hours at 37°C to establish bacterial cultures of roughly equivalent concentrations. These plates were then i) spotted unto carb+LB agar plates (Nunc #212811), grown overnight at 37°C and stored at 4°C to establish a "hard copy;" ii) replated (2x) into 8% glycerol LB+ antibiotics, grown overnight at 37°C, sealed and frozen at -80°C as a stock/backup; or iii) replated and grown overnight for use as a starter plates for feeding cultures.

The resulting nuclear sublibrary containing 738 RNAi clones targeting 650 genes. With empty vector controls, the library occupies eight 96 well plates. The complete list of genes targeted by RNAi, RNAi locations, rearray information, microarray expression data, and previously described phenotypes are available on the server/supplemental.

Background mutations

The screen was performed in worm strains carrying mutations in three key transcriptional silencing pathways i) Polycomb/H3K27me3, ii) SuVar3(9)/H3K9me2/3, and iii) heterochromatin protein 1 (HP1). The following strains were used: SS222 (*mes-3* (*bn21*) I (ts)); MT13293 (*met-2*(*n4256*) III); and PFR61 (*hpl-1*(*tm1624*) X; *hpl-2*(*tm1489*) *unc-49*(*e407*) III (ts)).

The *mes-3* (*bn21*) allele is temperature sensitive, strict maternal effect sterile at 25°C (Xu et al., 2001). The temperature sensitive period has been identified as embryogenesis and larval development, and the progeny of homozygous mothers, raised at the restrictive temperature, are 100% sterile (Xu et al., 2001). At 15°C homozygous *mes-3* (*bn21*) worms display a severe mortal germ line effect, leading to progressive

sterility in five to seven generations (Strome lab, pers. communication, Senchuk et al., unpublished). This allele corresponds to single base pair and is not a null mutation, but leads to protein destabilization and mislocalization of the MES-3 to the cytoplasm, an effect observed at 15^{o}C and much more severe at 25^{o}C (Xu et al., 2001). By immunofluorescence performed in M-Z- embryos at 25^{o}C, H3K27me3 staining is severely reduced, consistent with previously published reports of MES/PRC2 function (Bender et al., 2004).

The *met-2*(*n4256*) allele represents a 1343 bp deletion of a 5552 bp transcript, and removing portions of exon three, all of exon four, and the start of exon five, and is presumed to be functionally null (Andersen & Horvitz, 2007). This strain also has a mortal germ line defect, although much less severe than that observed in *mes-3* mutants, becoming sterile after 18-28 generations (Andersen & Horvitz, 2007). In agreement with most data currently available, *met-2* (*n4256*) embryos in our hands show a dramatic decrease in H3K9me2, while H3K9me3 is only marginally affected (Bessler et al., 2010).

The phenotypes associated with *mes-3* and *met-2* (H3K9me2/3, mortal germ line) allowed for identification of genetic enhancers, where loss of a gene by RNAi mediated knockdown results in a more severe phenotype (i.e., sterility, embryonic lethality). As both *mes-3* (*bn21*) and *met-2* (*n4256*) strains will produce F_1 progeny, these strains could not be used to identify genetic suppressors.

HPL-1 and HPL-2 encode partially redundant heterochromatin 1 (HP1 proteins). The *hpl-2*(*tm1489*) (ts) allele mutation starts upstream of the start codon and removes all of the first and second exons, which includes the chromodomain (Coustham et al., 2006). The *hpl-1*(*tm1624*) allele deletes 1700, deleting exons two through and resulting in a

premature stop codon. This allele is likely null, as most of the chromodomain and the entire chromoshadow domain are absent (Schott et al., 2006). Worms carrying both mutations (*hpl-1; hpl-2*) and raised at the restrictive temperature (25^{o}C) give rise to progeny that arrest early in larval development (L2), a phenotype allowing for identification of both enhancers and suppressors. Phenotypes associated with this strain are temperature dependent; at 24^{o}C *hpl-1; hpl-2* worms arrest as L3-adult worms with a synthetic multivulva phenotype (Muv).

Plate preparation

To screen worm populations, the 96 well format was rearrayed to 24 well format (Cell Star # 662102). Standard NGM media was prepared and supplemented with IPTG (to a final concentration of 5 mM), carbenicillin (final concentration = 50 ug/mL), and tetracycline (final conc. = 12.5ug/ml). An automatic plate pourer was used to add one ml/well. Plates were dried until solid, and seeded with bacteria within 12-48 hours.

Bacterial feeding cultures were started one day prior to seeding by growing an overnight culture. This overnight culture was replated into fresh LB + carb (150ul), and grown for 8 hours in a humidified shaker (37^{o}C, rpm <200). From this 8 hour culture, 10 ul feeding culture was spotted to each well using a multichannel pipette. Two or more replicate plates were made from a single RNAi culture prep. Plates were stacked and covered with worm tupperware to avoid cracking and drying of the agar (outer corners most susceptible). Bacterial culture was allowed to dry/grow for 2 days at 22^{o}C (RT), and seeded with worms immediately or stored at 4^{o}C for less than one week before use.

Preparation and seeding of worms

The mutant worms were grown up on multiple (five to fifteen, depending on the strain) large NMG plates containing OP50 bacteria at 15°C until the majority of worms were gravid hermaphrodites. Worms were washed off plates with M9 and bleached for three to four minutes with constant agitation, washed three times and resulting embryos were plated with minimal liquid unto one to two unseeded plates (no food) and allowed to hatch at 15°C overnight. Under these conditions, larvae undergo a reversible developmental arrest as early larvae. The next day, synchronized L1 larvae were rinsed from the unseeded plate. Concentration was adjusted to one to two worms/ul. Three ul of worms were added to each well, and the presence of three to six worms was verified using a dissection microscope. More worms were added per well for *mes-3*(*bn21*) strain (eigth to ten) to account for the severe mortal germ line defect and avoid a false positive "steriles."

The plates were then shifted to 25°C and incubated for seven to nine days to allow for complete development of F_1 progeny. As P_0 *hpl-1; hpl-2* worms shifted to restrictive temperature were found to arrest without producing progeny, screening plates seeded with worms were incubated at 15°C (permissive temperature) for 24 hours before shifting to 25°C; at this time, *hpl-1; hpl-2* worms are still L1 larvae.

Screening and scoring

Phenotypes of the F_1 progeny were scored using a standard dissection scope. To avoid bias, scoring was performed blindly, without knowledge of the RNAi target. A schematic representation of the screen and the phenotypes score is shown in Figure 5.

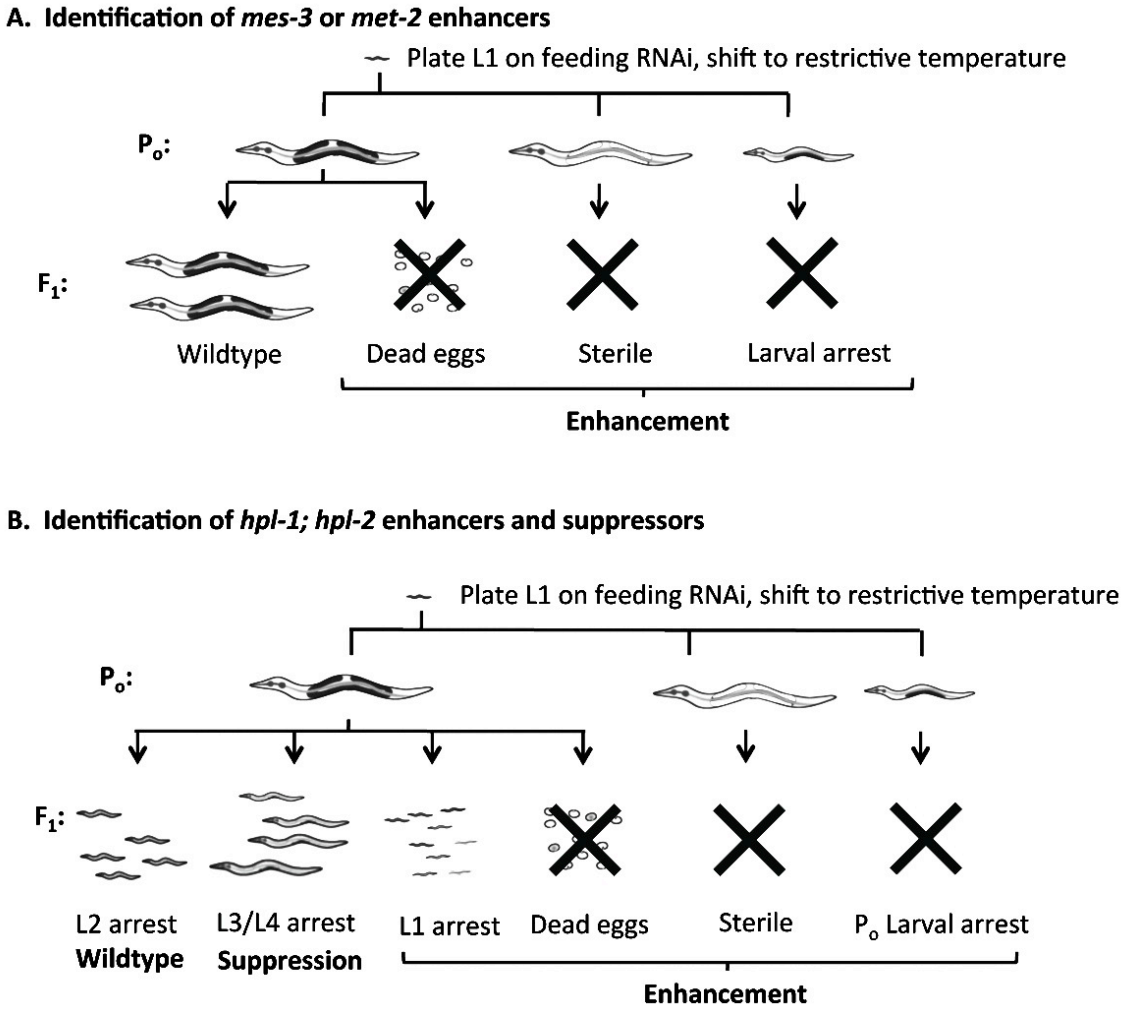


Figure 5. Schematic representation of the primary screen.
All mutant strains [*mes-3(bn21)*, *met-2(n4256)*, and (*hpl-1*(*tm1624*); *hpl-2*(*tm1489*)] were screened by seeding the Po generation as synchronized L1s, and shifted to restrictive temperature (25^{o}C). F1 progeny were examined after 7-9 days. The top panel (A) shows the phenotypes associated with mes-3 and met-2 enhancement. Po were observed to arrest as larvae (L1-L4), sterile adults, or dead eggs. Larval arrest of F1 progeny was not observed. The bottom panel (B) depicts enhancement and suppression phenotypes associated with *hpl-1; hpl-2*. Similar enhancement phenotypes were observed, as well as early arrest of F1 larvae. Suppression is characterized by growth past the L2 stage.

Enhancement of the three different mutant strains could be divided into three classes: 1) larval arrest of P_0, defining the strongest category of enhancement; 2) sterility of P_0; and 3) fertile P_0 but dead embryos or developmentally arrested F_1 progeny. Categories 2 and 3 are classified as enhancement, although category 3 is considered appreciably weaker. As this class of enhancers is quite small, categories 2 and 3 were grouped during further analysis.

Suppression of the *hpl-1; hpl-2* double mutant was characterized as growth beyond the L2 stage, and was classified as "strong" if more than ~50% of F_1 animals grew past L3, or more than ~20% grew past L4 and/or developed a protruding vulva, indicative of late stage developmental events. Controls never demonstrated this phenotype, and *mes-2* positive controls were consistently strong suppressors.

Phenotypes associated with RNAi in mutant strains were compared to i) the replicate test (reproducibility), ii) the published RNAi phenotype(s) annotated in Wormbase, and iii) the phenotype observed in replicates tests of the other mutant strains.

Results and Discussion: Primary screen

In this work, mutants involved in transcriptional silencing were screened against a sublibrary containing 738 RNAi clones targeting 650 genes associated with nuclear localization/function or RNAi mechanisms by GO term annotation. 577 RNAi clones targeting 511 genes either enhanced or suppressed one or more of the three mutant lines at some level in both replicates. These can be separated into different categories based on phenotypes observed in each mutant; genes associated with different categories are

plotted in Figures 6 and 7. Categories were subdivided further based on previously annotated RNAi phenotypes (data not shown).

Of the 577 potential positives, 284 were found to enhance all three mutations; the majority of these had previously identified RNAi phenotypes associated with sterility, embryonic, larval, or adult lethality, which likely account for the observed phenotype. 18 genes in this category are not associated with previously annotated RNAi phenotypes, and 12 of these 18 genes were tested in an N2 wildtype background. In N2, 5 of 12 RNAi treatments resulted in phenotypes similar to those observed in the mutant strains during screening, suggesting that at least some genes in this category represent a general, but not previously annotated phenotype; these phenotypes are not associated with enhancement. Phenotypes resulting from the remaining RNAi (7/12) may represent general enhancers (i.e., sickly or slow and likely to enhance many mutations), although this was not tested.

We identified 293 RNAi clones targeting 268 genes that are potentially interesting candidates to function in heterochromatin formation and/or transcriptional repression. Many of these genes enhance and/or suppress more than one background mutation, suggesting that a great deal of crosstalk exists between these different silencing mechanisms. For example, 102 genes enhanced the *met-2* mutation, but less than half of that number exclusively affected *met-2*. Similar overlap was seen between each of the background mutations.

Next, we queried for specific enrichment of GO terms within each of these categories relative to the entire sublibrary using the GOstat online tool developed by Tim Beissbarth (http://gostat.wehi.edu.au/cgi-bin/goStat.pl). Intriguingly, two categories i)

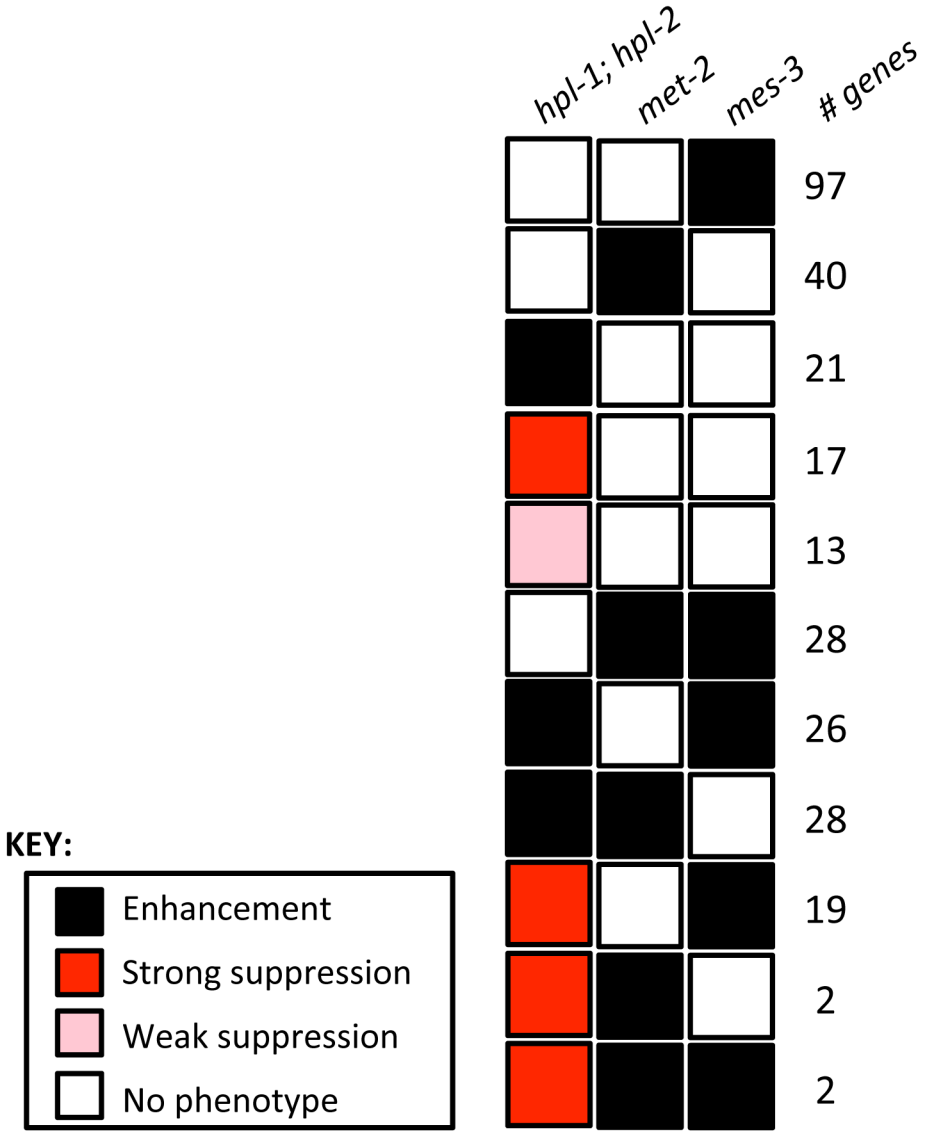


Figure 6. Summary of primary screen results. The different mutant backgrounds are represented by individual columns, and different phenotypes correspond to the different colors; red indicates enhancement, black indicates strong suppression, grey indicates weak suppression, and white indicates no phenotype. The number of genes associated with the different phenotypes is shown at the right.

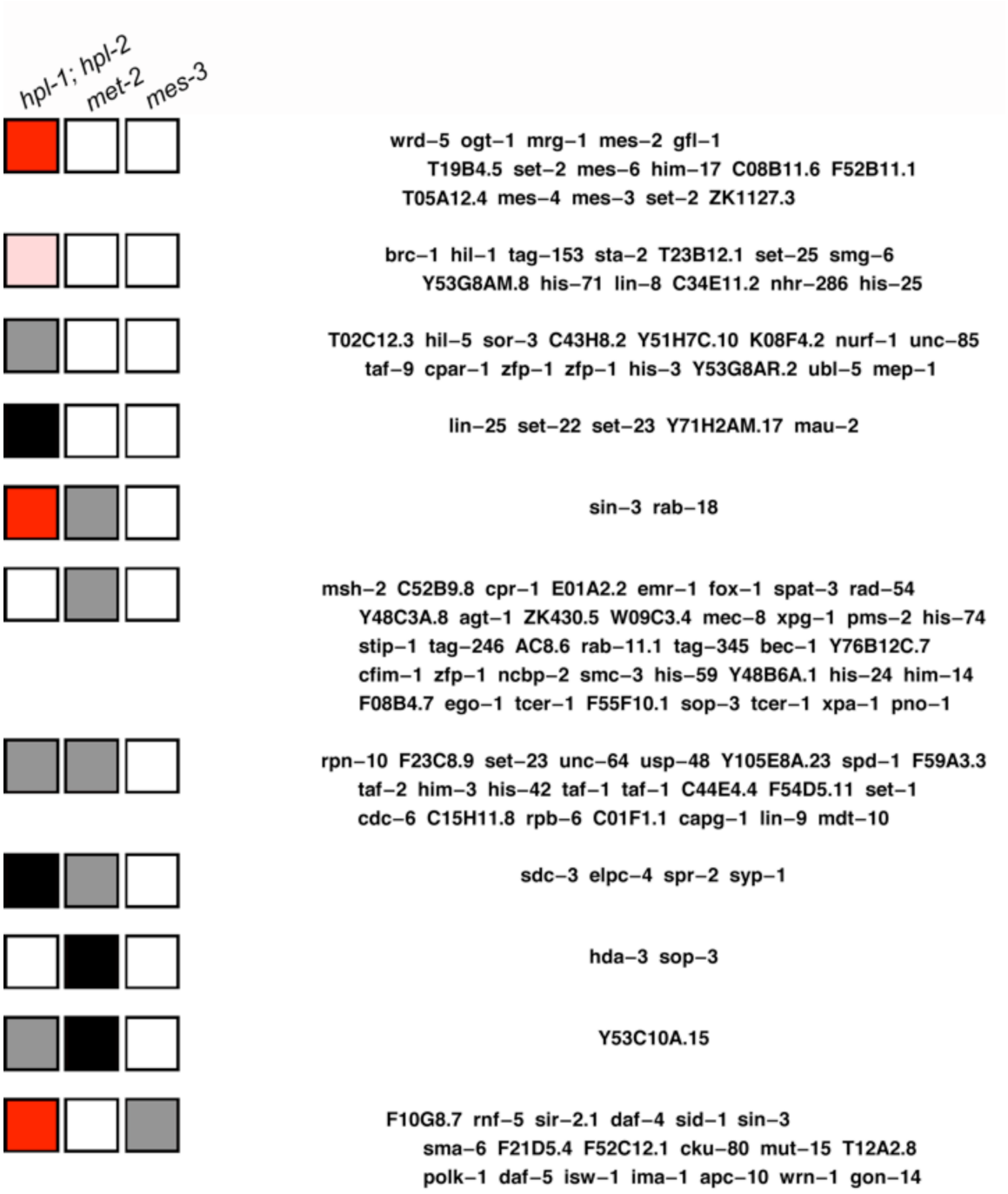


Figure 7. Breakdown of primary screen results. Genes are plotted by phenotypes associated with different background mutations. Red indicates strong suppression and pink indicates weak suppression. Black indicates strong enhancement, and grey indicates weak enhancement. Within the sublibrary, some genes were targeted by multiple RNAi clones, and are repeated in this analysis. Genes that weakly enhanced all mutant backgrounds, and genes that showed no phenotype in any background are not shown in this plot.

mut−16 mbtr−1 mbtr−1 mbtr−1 nhr−244 brd−1 mus−81 nuc−1
C12D8.1 eor−2 hcf−1 C49H3.9 C56G2.7 cyn−8 sin−3
rtel−1 F28H6.4 dog−1 pmk−3 F45G2.3 sir−2.3 rsd−2 sta−2
mboa−2 zhp−3 mys−2 cmk−1 K07C5.3 set−12 mtss−1
nth−1 R74.6 unc−2 T05A12.4 brc−2 eif−3.K nhr−284
mdt−1.2 unc−83 set−19 polq−1 taf−6.1 nhr−235 pph−5
rab−35 msh−6 jmjd−2/KDM4 Y48C3A.14 smg−7 flt−1
pqe−1 cec−1 rad−23 lin−49 spd−1 spd−1 W04A4.5 pqn−85
Y39A1A.14 klc−2 set−4 his−35 F54E12.2 F56A8.3 mei−2
pms−2 M03C11.2 unc−116 cki−2 atl−1 sut−1 dpy−28
smk−1 sgo−1 bir−1 Y61A9LA.10 xrn−1 smk−1 K04C2.2
drh−3 tsn−1 msh−4 tag−203 psa−4 eya−1 F58A4.9 F59A7.8
eri−1 eri−1 ubc−1 T19B10.6 hpl−2 lin−59 rad−51 npp−1

spr−5 set−6 C56E6.2 smg−4 Y108G3AL.7 prmt−1 Y71G10AL.1
crn−1 lin−54 dpy−26 lig−1 mdt−22 his−66 him−14
F32B6.3 rfc−2 taf−10 gon−4 let−49 Y47D3A.29 rsp−7

tax−6 vhp−1 mom−2 his−39

suf−1

set−25

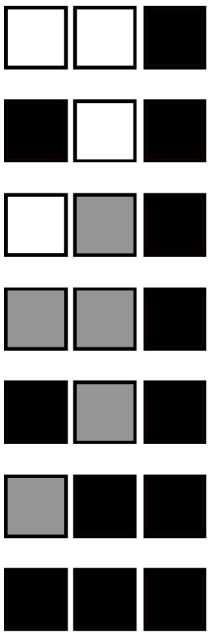
rsp−6 C44B7.2 ttll−12 T13F2.2 chd−3 pme−1 exo−3 taf−1 sop−3
mtk−1 npp−5 F32E10.6 spt−4 K10D2.1 rec−8 his−38 xrn−1
rab−10 nol−10 rfc−1 spo−11 dpy−30 hrp−2 F27C1.6 vps−34

his−10 his−26 Y47D3A.29 cpsf−2 npp−8 npp−8
npp−8 npp−8 xpo−1 his−9 his−7 psa−1 ruvb−1 imb−3
his−31 snfc−5 Y76B12C.7 his−37 mfap−1 his−67
D2096.8 epc−1 mdt−8 his−56 his−57 his−61 skr−1
nst−1 npp−8 his−64 his−32 let−526 pqn−85 pro−2

sop−3 sop−3

mag−1 tag−214 scc−1 W03F9.10 Y23H5B.5

Figure 7. Continued.



siah−1 Y116A8C.13 ufd−2

dre−1

din−1

mcm−7 emb−5 scc−3 fib−1 E02H1.1 npp−9 T01B7.5

his−12 uaf−1 npp−10 hda−1 xpo−2

xrn−2

mif−3 dhp−1 Y77E11A.6 rpt−6 his−16 his−34 his−44 his−44 his−44
his−43 dhc−1 rpt−4 his−65 his−47 his−13 rpb−2 rpt−3
vha−11 hel−1 F43G9.12 rab−1 imb−3 his−63 ama−1 his−55
skp−1 xpo−2 prp−8 sca−1 let−858 K03H1.10 Y46G5A.4 snr−7
rpt−2 his−58 rpn−6 prp−21 rpt−5 T13H5.4 dre−1 his−6 spt−5

Figure 7. Continued.

hpl-1; hpl-2 suppression, *mes-3* enhancement, and ii) *met-2* enhancement, demonstrate enrichment of factors associated with the GO term "DNA repair." Other categories failed to show significant enrichment, likely due to the number of genes in each category being relatively low. By manual analysis of GO terms, 32 of the 52 DNA repair associated factors in the RNAi sublibrary are among the 268 candidate genes, suggesting that DNA repair may be linked to heterochromatin formation.

We next questioned whether components of known complexes would track together within any of the categories, indicating a genetic interaction between a complex and transcriptional silencing pathway(s). Complexes examined included those involved in chromatin remodeling (SWR1, NURD, etc.), transcriptional activation/repression

(DREAM, SAGA, COMPASS, etc.), and complexes involved in DNA damage and repair, meiosis, dosage compensation, protein degradation, and RNAi. This analysis was performed manually, against predicted *C.elegans* homologs. As subunits of these complexes have not been verified *in vivo* and/or studied in *C.elegans*, many predicted orthologs were not annotated by GO terms, and thus not in the sublibrary used for screening – this represents one of the major drawbacks of the bioinformatics based approached used.

Using this comparative method, all known members of the Polycomb Repressive Complex 2 (PRC2) (*mes-2, mes-3, mes-6*) and two interacting factors (*mes-4, set-2*), are found in the "strong *hpl-1; hpl-2* suppressor" category, indicating that the combined loss of *mes* gene function and *hpl-1; hpl-2* mutation causes a significantly less severe phenotype than *hpl-1; hpl-2* alone. In the conditions used for this screen, progeny of *hpl-1; hpl-2* mutants (i.e., F_1 animals) arrest as early larvae (L1-L2). Loss of *mes* gene function in this background strongly suppressed the early arrest, and fit the criteria for "strong suppression," with ~50% of F_1 animals grew past L2 or more than ~20% grew past L4 and/or developed a protruding vulva, indicative of late stage developmental events. No other complex or pathway shows such a striking pattern of enrichment within a single category. Although we have not ruled out interactions between the Mes pathway and HP1, this distinctive signature may allow for the identification of novel factors functioning in collaboration with Mes genes in the repression of X-linked expression, chromatin dynamics, and the regulation of the transition between cellular plasticity and cell fate specification.

Discussion

The nuclear/RNAi candidate screen described in the previous sections was carried out to identify factors functioning in heterochromatin formation. To accomplish this, the candidate RNAi sublibrary was screened using strains carrying background mutations in three conserved pathways mediating transcriptional repression: *mes-3* (Polycomb), *met-2* (H3K9me2) and *hpl-1; hpl-2* (HP1). As loss of these components have subtle phenotypes, we hypothesized that additional methods of gene silencing likely exist, and that the combined loss of these unidentified components with known mediators of transcriptional repression would have additive effects.

We were most intrigued by the observation that loss of all known MES/PRC2 components (*mes-2, mes-3, mes-6)*, as well as RNAi's targeting two genes shown to genetically interact with the PRC2 complex *(mes-4* and *set-2*), strongly suppressed the *hpl-1; hpl-2* larval arrest phenotype, and failed to enhance *met-2* and the *mes-3* loss of function phenotypes. SET-2 is enriched in the PGCs of L1s and the germ cells of adults, similar to the MES factors, and has been shown to enhance the sterility of *mes-3* and *mes-4* M+Z- mutants (Xu & Strome, 2001). Although SET-2 is required for H3K4me2/3, these marks are absent in the PGCs (Schaner et, al, 2003), removal of which may be dependent on MES-4 and the demethylase SPR-5 (Furuhashi et al., 2010; Katz et al., 2009). *set-2* also has a mortal germ line, lending further support for SET-2 function with MES factors in promoting germ line function (Xiao et al., 2011).

As SET-2 was initially identified as part of the Mes/PcG pathway as an enhancer of *mes-3* and *mes-4* sterility, we might have predicted that additional components of this pathway might also enhance *mes-3* sterility. However, under the conditions used for our

screen, *set-2* did not enhance *mes-3*, but rather suppressed *hpl-1; hpl-2*, suggesting that the knockout of *mes-3* using the temperature sensitive allele is sets a high threshold, such that it cannot be enhanced further by components functioning in a common pathway. This is supported by the observation that all F_1 animals failed to give rise to an F_2 generation. In addition, our findings validate a previous result demonstrating that *mes-2*, *mes-4* and *set-2* suppress the *hpl-1; hpl-2* phenotype (Simonet et al., 2007). However, this group did not explore the mechanisms by which this suppression occurred, nor address the potential link to the Polycomb pathway.

Two complexes (SOP-2/SOR-1/SOR-3 and SPAT-3A/MIG-32) have been postulated to form PRC1 like complexes in *C.elegans*. The defects associated with loss of function of these genes are associated with the subtle homeotic transformation phenotypes linked to misregulation of *Hox* genes. Whether these complexes are PRC1-like, and whether they interact with *mes-2/3/6* is controversial. As shown Table 1, these genes do not suppress *hpl-1; hpl-2* larval arrest, suggesting they are unlikely to function in the MES pathway of chromatin regulation. However, interaction between these genes (*sop-2/sor-1/sor-3* and *spat-3A/mig-32*) and the MES factors during the regulation of *Hox* genes is not ruled out.

It is possible that the observed rescue of *hpl-1; hpl-2* larval arrest by MES factors is due to interaction with the SynMuv related role of *hpl-2*. Several studies have shown that loss of *mes-4 (*as well as *mes-2/3/6* in the case of *lin-15AB(n765)*), suppresses the Muv phenotype associated with SynMuv mutation (Cui et al., 2006; Lehner et al., 2006; Simonet et al., 2007; Petrella et al., 2011), and *mes-2/mes-3/mes-6* and *mes-4* have been shown to suppress some SynMuv associated phenotypes, including the expression of

Table 1. Phenotypes associated with predicted components of *C.elegans* PRC2 and PRC1.

	Gene	Domains/Homology	Supp.:	Enhancement:		
			hpl	*hpl*	*mes-3*	*met-2*
PRC2	*mes-2*	E(z)/EZH2, SET (H3K27me)	+++	-	-	-
	mes-3	Novel	+++	-	-	-
	mes-6	ESC/EED	+++	-	-	-
	mes-4	SET (H3K36me), PHD	+++	-	-	-
	set-2	SET (H3K4)	++	-	-	-
PRC1 (?)	*spat-3*	Ring1 ZF, H2Aub	-	-	-	-
	mig-32	Ring, H2Aub	n.d.	n.d.	n.d.	n.d.
	sor-1	Novel	-	+	+	-
	sor-3	Novel, MBT	-	+	-	-
	sop-2	SAM domain	-	-	-	-

ectopic P-granules (Unhavaithaya et al., 2002; Petrella et al., 2011). However, this effect is unlikely to be due solely to suppression of SynMuv vulval phenotypes, as a number of RNAi clones resulting in strong *hpl-1; hpl-2* suppression in this screen (including *mes-2, mes-3,* and *mes-6*) were characterized by well developed worms in which multiple vulvas were apparent. This observation is explored further in the secondary screens section.

In addition to members of MES pathway/Polycomb repressive complex, 11 other genes were found to strongly suppress *hpl-1; hpl-2* with no effect on *mes-3* or *met-2*, raising the interesting hypothesis that genes with this signature may represent additional genes functioning in the Polycomb pathway. Table 2 provides a summary of these genes, a number of which are associated with different activating complexes, including NuA4, SWR1, and COMPASS. Function of these factors in cooperation with Mes mediated repression is somewhat unexpected, as these complexes are generally associated with gene activation, mediating histone acetylation, incorporation of the histone variant H2A.Z, and H3K4 methylation respectively.

Table 2. Strong *hpl-1; hpl-2* suppressors identified in the primary screen.

	Gene	Function	Homologs	Chr.	Domains
NuA4 / SWR1	mrg-1	Mortality factor-related protein, cell proliferation	MSL-3, MRG15, EAF3	III	Chromodomain, RNA binding activity
	ZK1127.3	Uncharacterized conserved protein	EAF7, MRGB	II	CT20
	gfl-1	Transcription initiation factor IIF, auxiliary subunit	Yaf9, Gas41, Yeats4, AF9	IV	YEATS
	C08B11.6	Actin-related protein, Arp6p	ARP6	II	Actin-like
COMPASS	set-2	Histone H3 (Lys4) methyltransferase complex	SET1/MLL	III	SET- domain
					SET domain, RNA recognition motif
	wdr-5	Histone H3 (Lys4) methyltransferase complex	WRD5, SWD3	III	WD40/YVTN
	F52B11.1	Histone H3 (Lys4) methyltransferase complex	SPP1/CFP1	IV	CpG binding, Zn Finger
					CpG binding, Zn finger
					n/a
Repair	him-17	Meiotic repair, H3K9me2	n/a	V	THAP
	T05A12.4	DEAD box-containing helicase-like transcription factor/DNA repair protein	RAD16	IV	DEAD helicase, SNF2-related, ZnF
					DEAD helicase, SNF2-related
Other	T19B4.5	Uncharacterized	n/a	I	Coiled-Coil
	ogt-1	O-linked N-acetylglucosamine transferase OGT	OGT	III	Tetratricopeptide repeat

NuA4 is a multisubunit complex participating in histone acetylation of H4 and H2A at target promoters, and as such, is typically thought to induce transcriptional activation. The SWR1 complex is an ATP dependent chromatin remodeling complex, involved in the incorporation of the histone variant H2A.Z, which is important for gene regulation and genomic stability. The incorporation of H2A.Z is thought to change nucleosome dynamics, but the effects of these changes on gene expression have been controversial (Marques et al., 2009). In yeast, at least four components are shared between these two complexes, and evolutionary evidence suggests that overlap may be greater in complex metazoans (Lu et al., 2009). Four genes predicted to function in the SWR1/NuA4 complex were identified as strong *hpl-1; hpl-2* suppressors: *mrg-1*, *gfl-1*, *C08B11.6* and *ZK112.73*. The mixed lineage leukemia (MLL) protein and the *Drosophila* homolog, Trithorax, exist in COMPASS (complex of proteins associated with Set1)-like complexes and mediate H3K4 methylation. As shown in Table 2, several genes associated with the COMPASS complex were identified as *hpl-1; hpl-2* suppressors, including the H3K4 HMT *set-2*, a previously identified Mes interactor, as well as *wrd-5*, and *F52B11.1*. MLL/COMPASS mediated marks form the positive component of "bivalent" domains thought to mark gene poised for activation during differentiation; this may represent one means of interaction with the Mes pathway.

In addition to components of the transcription activating complexes noted above, two genes potentially involved in DNA repair were identified as *hpl-1; hpl-2* suppressors, including *him-17* and *T05A12.4*. HIM-17 is also required for the proper patterning of H3K9me2 marks in the meiotic germ line (Reddy & Villeneuve, 2004; Maine et al., 2005; Bessler et al., 2007). A gene encoding O-linked N-acetylglucosamine (O-GlcNAc)

transferase (OGT-1) was also found to suppress *hpl-1; hpl-2*, and as this modification may be linked to Polycomb function in flies and mammals (Love et al., 2010), we were intrigued to discover a link between OGT and the Mes/PcG pathway inthe worm. The last *hpl-1; hpl-2* suppressor is *T19B4.5*, which has been previously linked to the RNAi pathway (Wang et al., 2005), but appears to be novel, uncharacterized, and by sequence analysis may not be conserved in other species.

Many of the *hpl-1; hpl-2* suppressors identified by this study are generally involved in the positive regulation of gene expression. In the most simplistic model, the loss of these factors (and likely complex functionality) may suppress *hpl-1; hpl-2* larval lethality by "balancing out" the loss of these repressors. One could imagine that in *hpl-1; hpl-2* mutants, lethality results from overactive transcription and/or the activation of off-target genes. By inhibiting precocious gene activity by removing one mode of gene activation, it is possible a favorable equilibrium of gene expression might be reestablished, allowing for enhanced developmental potential.

There are two main reasons to argue against this hypothesis. First, the MES/PRC2 and associated factors MES-4 and SET-2 represent 5 out of the 16 factors whose loss leads to suppression of the *hpl-1; hpl-2* phenotype. As PRC2 is appreciated to function primarily in transcriptional repression, the simple model presented above does not hold up. Secondly, in both worms and other organisms, the understanding of HP1 function is increasingly complex. For example, recent studies also implicate HP1 in processes such as DNA repair and euchromatic gene expression (Kwon & Workman et al., 2011). HP1 has also been associated with both positive and negative regulation of PolII elongation (Vakoc et al., 2005; Milne et al., 2005). Similarly, HP1 has been

associated with both heterochromatin and euchromatic regions of *Drosophila* polytene chromosomes (Kwon & Workman, 2011). As these factors are both transcriptional repressors, an alternative model suggests that HP1 function might oppose MES/PcG during the transition from cellular plasticity to differentiation (for example, HP1 might inhibit differentiation genes while Mes/PcGs inhibit pluripotency).

In worms, immunostaining has demonstrated that HP1 localized regions lie immediately adjacent to condensed chromatin, but were found not to overlap with condensed domains (Couteau et al., 2002). This finding suggests that HP1 in *C.elegans* may function somewhat differently than other organisms. In addition, numerous studies in a range of species has demonstrated that HP1 binds H3K9me2/3, but this link has not been established in *C.elegans*. In fact, HP1 foci do not appear to overlap with either di or tri methylated H3K9, and in differentiated adult nuclei, HPL-2 staining is diffuse (Simonet et al., 2007). However, in *hpl-2* mutants, H3K9me3 appears to become more concentrated in nuclear foci (Simonet et al., 2007), suggesting that HP1 might function to stabilize certain chromatin configurations. As MES-2 is essential for H3K9me3 in the germline, this may provide a link between the MES factors and HP1, despite the lack of evidence for direct binding of HP1 to this mark. Intriguingly, the localization of H3K9me3 in *hpl-2* mutants is restored to wildtype in *hlp-2; set-2* double mutants (Simonet et al., 2007). These data support the findings of our study, which suggest that while the activities of MES-2/PRC2 and SET-2 may be in opposition (i.e., repression vs. activation), these factors work together to ensure correct expression of germ line gene profiles. It is possible that the role of HP1 is actually to antagonize or erase the germline

chromatin state, potentially through removal of MES-4 mediated H3K36me, and in this way functions in the SynMuv pathway.

To begin to define the roles of the *hpl-1; hpl-2* suppressors identified by our primary screen, specifically with respect to the Mes/PcG pathway, we tested whether loss of these factors share similar MES related phenotypes, including maternal effect sterility in wildtype worms, loss of H3K27me3 staining during embryogenesis, and rescue of *mep-1*/NuRD associated larval arrest. We hypothesized the different *hpl-1; hp-2* suppressors identified in our primary screen might function more similarly to either MES/PRC2 or MES-4. Loss of MES-4 has been associated with more severe defects than loss of MES-2/MES-3/MES-6, including the germ line degeneration phenotype (Capowski et al., 1991, Garvin et al., 1998). MES/PRC2 complex localization and function is distinct from MES-4, although these genes function in a common pathway to repress transcription of the X chromosomes in the adult germ line. While the PRC2 complex associates with all chromosomes and trimethylates H3K27, MES-4 localizes to autosomes and mediates H3K36me2/3 methylation, a mark correlated with transcriptional activity, as well as recruitment of histone deacteylase complexes (Fong et al., 2002; Bender et al., 2006; Rechtsteiner et al., 2010; Furuhashi et al., 2010; Leib & Clark, 2005). Studies have also indicated that these genes may possess some independent functions, including repression of RNA PolII elongation in the PCGs, and regulation of chromatin compaction and gene expression during the transition from cellular plasticity to cell fate commitment (Furuhashi et al., 2010; Yuzyuk et al., 2009). Identification of additional factors primarily affiliated with MES-4, as well as factors functioning predominantly with MES/PRC2 will allow for a greater understanding of the Mes as a whole, and

perhaps shed light on the mechanisms mediating MES antagonism, targeting, and regulation of heterochromatin.

Note on interpretation of results

It should be noted that the setup and analysis of this screen may contain a certain percentage of "false negatives." As such, we may have overlooked some potentially significant factors. One example is H1.1 (*his-24*), a linker histone thought to be involved in heterochromatin formation (Jedrusik & Schulze, 2001; Jedrusik & Schulze, 2007). Loss of *his-24* showed no significant phenotypes in *hpl-1;hpl-2* or *mes-3* mutant strains, but "enhanced" *met-2*. There are several possibilities that may account for this observation. First, as *his-24* RNAi is previously associated embryonic lethal and sterile RNAi phenotypes, it is possible that *his-24* RNAi in this screen actually suppresses both *hpl-1; hpl-2* and *mes-3* and but has no affect on *met-2* other than the previously identified phenotype. An important alternative is that this RNAi clone was only weakly effective, resulting in a generally mild phenotype. It is also possible, based on the inherent properties of the RNAi libraries, as well as human error, that some clones may be incorrect, and/or contaminated with other RNAi clones. 70/493 clones expected to show an embryonic, larval or adult lethality or arrest had no effect in any of the three mutant lines; it is likely these are weak or ineffective RNAi's. In addition, 54/493 clones with previously annotated phenotypes affected only a single mutant line. However, it must also be considered that previously annotated RNAi phenotypes may be overstated, as these data are culled from many types of experiments, including different background

mutations (including RNAi enhancer strains), and may reflect partial or not fully penetrant phenotypes.

Yet based on previous studies, one might have predicted that *his-24* would function in the MES/PcG pathway. Loss of *his-24* function is associated with desilencing of repetitive transgenes in the germ line, germ line proliferation defects and low level sterility, phenotypes commonly associated with loss of MES activity (Jedrusik & Schulze, 2007). However, as *his-24* loss of function enhanced H3K27me staining in *mes-3 (bn35)* germline nuclei (Jedrusik & Schulze, 2007), we would not predict that *his-24* would suppress *mes-3*, as in the alternative interpretation of our results. Yet, as the ascribed role for this protein germ line is dependent on *mes-2, mes-3, mes-4, mes-6* and *sir-2.1* (Jedrusik & Schulze, 2007), it's possible that factors functioning downstream of MES activity have different effects on *hpl-1; hpl-2* and/or other mutant backgrounds.

Additional data and potential future directions

This screen provides a wealth of data not explored in this manuscript. Each of the categories of enhancement/suppression within the different mutant strains may provide insight applicable to future studies. Of specific interest might be the class associated with both *hpl-1; hpl-2* suppression and *mes-3* enhancement, as these factors may function in some capacity within the MES/PcG pathway. As shown in Figure 7, this group contains predicted DNA binding proteins (R07E5.8, F21D5.4), histone deacetylases (SIR-2.1, SIN-3) and SET domain containing proteins (SET-12, SET-19). These types of factors could be hypothesized to function in the MES/PcG pathway of silencing, and may represent interesting targets of future study.

A second area that may be of interest is the potential connection to DNA repair. A total of 90 genes in the *C.elegans* genome are annotated with the GO term DNA repair. Of these, 52 are present in the nuclear sublibrary. A striking 45/52 DNA repair associated genes enhance or suppress one or more mutant strains. While 12 of these enhance all three mutants, and may represent a general enhancers and/or previously annotated RNAi phenotypes, the enrichment of DNA repair factors is intriguing, as DNA repair machinery is increasingly implicated in transcriptional regulation, and may also play a role in the transition from pluripotency to differentiation. One striking example comes from the Tjian lab, where it was recently demonstrated that the stem cell coactivator complex (SCC), which interacts directly with Oct4 and Sox2, binds Oct4 and Nanog promoters, and is required for both maintenance of ES cell pluripotency and differentiation potential is actually the XPC nucleotide excision repair complex (Fong et al., 2011). Although the mechanism is unknown, perhaps a similar complex functions in *C.elegans* embryogenesis.

In addition, a growing body of work demonstrates that chromatin factors, including Polycomb, are recruited to sites of DNA damage and may function to block transcription of the damaged elements and/or remodel the surrounding chromatin environment to allow access of DNA repair machinery (Bell et al., 2011). We are only beginning to appreciate how the interplay between DNA surveillance and repair are coupled with transcription, particularly with respect to the pluripotent cell population. To quote from a recent review:

"Understanding the contribution of Polycomb group proteins to the DNA damage response may lead to novel therapeutic strategies that increase the response of human

cancers to therapies that work through DNA damage, while simultaneously sensitizing the cancer stem cell population that would otherwise lead to relapse" (Gieni et al., 2011, pg 883).

Factors associated with DNA repair were also enriched in the *met-2* enhancer category. This may reflect recent findings demonstrating a role for MET-2 in meiotic sex chromosome inactivation (MSCI) and shielding the unpaired X chromosome from recognition and triggering of checkpoint activation (Checchi & Engebrecht, 2011). In this study, *met-2* XO animals undergo increased germ line apoptosis, due to inappropriate activation of the recombination checkpoint. Intriguingly, MES-2 activity also appears to be required in this process, supporting the observation that crosstalk likely occurs between silencing pathways (Checchi & Engebrecht, 2011; Senchuk et al., unpublished).

Methods: Secondary screens

hpl-1; hpl-2 suppression assay: Larval arrest

The *hpl-1; hpl-2* strain used in the previously described screen [PFR61, *hpl-1(tm1624)* X; *hpl-2(tm1489)*; *unc-49(e407)* III (ts)] was used to confirm and quantitate the level of suppression in response to RNAi mediated knockdown of the different clones identified as *hpl-1; hpl-2* suppressors in the primary screen. RNAi cultures were grown using sequenced clones in 5 ml LB + 50 ug/ml carbenicillin for 8 hours at 37^{o}C. RNAi plates were prepared using 5 cm NGM plates, seeded with 1 ml of cultured bacteria resuspended in 100 ul of IPTG and carbenicillin to a final plate concentration of 8 mM and 60 ug/ml respectively. Seeded plates were grown for 48 hours at RT/22^{o}C, and

stored for up to a week at 4°C. This method of plate preparation was used throughout the secondary screening analyses.

hpl-1; hpl-2 worms were maintained at 15°C, and synchronized as L1s by bleaching a population of healthy, gravid adults and seeding embryos on unseeded NGM plates. For each assay, 10 L1s were transferred to individual RNAi plates. These were incubated at 15°C (permissive temperature) for 24 hours to avoid P_0 arrest. Plates were then shifted to 25°C (restrictive temperature) for 8-9 days to allow progeny to fully develop. All resulting progeny were counted and scored as L1, L2, L3, L4 or adult based on size under dissection microscope. Staging was verified by examining germline development in subsets of worms by Nomarski optics under high power magnification. Using RNAi plates prepared using different cultures and different populations of worms, three or more replicates were performed per RNAi.

***hpl-1; hpl-2* suppression assay: Multivulva formation**

This assay was performed as above, but plates were shifted to the semirestrictive temperature of 24°C instead of 25°C to allow for all worms to reach maturity. F_1 worms were scored for the presence of multiple vulvas after 9-10 days. Three biological replicates were analyzed per RNAi.

Maternal effect sterile

Gravid N2 wildtype hermaphrodites were bleached and embryos were hatched in the absence of food to synchronize L1s. Ten-twenty L1s were moved to RNAi plates (prepared as above) and raised at 25°C. Late hatched L4 F_1 progeny (and thus most

strongly affected by RNAi) were moved to fresh RNAi plates and scored after 30-40 hours of development at 25°C. Adult worms were mounted on 4% agar pads in Levimiasole anesthetic and scored for sterility (i.e., absence of eggs or oocytes and/or endomitotic nuclei) at 5x and 20x. Approximatly one hundred worms were scored per RNAi treatment. Worms containing more than five wildtype appearing embryos were considered fertile.

Germline morphology

Germline morphology was assayed in wildtype N2 worms, as well as in worms carrying an integrated, germ line expressed H2B::GFP chromatin marker [AZ212 (unc-119(ed3) ruIs32[unc-119(+) pie-1::GFP::H2B] III)]. Synchronized L1 larvae were seeded unto RNAi plates, raised at 25°C until adulthood and either scored as P_0 (for maternal effect sterile genes) or allowed to lay embryos. A subset of F_1 progeny were moved to new RNAi plates and allowed to develop, and examined ~30 hours into adulthood. Fifty to one hundred animals per RNAi were mounted on 4% agar in Levimiasole and scored live. The size, shape, and relative number of germ line nuclei, as well as germ line organization in mitotic and meiotic zones were observed in comparison to control worms fed empty vector RNAi.

H3K27me3 staining

Changes in global H3K27me3 and H3K9me2 levels and localization patterns were assessed by immunofluorescence staining in fixed embryos following treatment with RNAi. Starved synchronized wildtype (N2) L1 worms were plated to either empty

vector or RNAi plates and raised 48-56 hours at 25°C, allowing the majority of animals to reach adulthood. Different incubation times were used as some RNAi treatments (including *gfl-1* and *T19B4.5*) result in slow growth in N2. Gravid adult were dissected on polylysine coated slides in M9, and older embryos were picked off the plate. Untreated embryos marked by integrated GFP were included to serve as on slide staining controls, these included: JR2132 (wIs126 (elt-7::GFP::lacZ)) and AZ212 (*unc-119(ed3)* ruIs32[unc-119(+) pie-1::GFP::H2B] III). Control stains included *mes-2* (RNAi) and *mes-3(bn21*) raised at 25°C. Coincident with wildtype (N2) experiments, RNAi plates from the same preparation were used to establish RNAi efficacy using *hpl-1; hpl-2* worms (i.e., suppression assay).

Slides were stained using standard protocols. Briefly, slides were freeze-cracked, were fixed for 10 minutes in 4°C methanol, washed 3x10 minutes in TBST, and blocked in TNB+NGS. Primary antibodies were diluted in TNB+NGS and incubated overnight at 15°C. The following primary antibodies were used in this analysis: rabbit anti-H3K27me3 (Millipore, #07-449, 1:500) and mouse anti-H3K9me2 (ab1220, abcam 1:500); these antibodies have been verified for specificity by the ModEncode Consortium and Egelhofer, et al., 2011. As a marker of on slide control embryos, chicken anti GFP (Milipore/Chemicon; #AB16901) was used at 1:500. Alexafluor secondaries were used at 1:200 (both mouse and chicken primary antibodies were detected using 488) and samples were mounted in SlowFade + Dapi (Invitrogen). Imaging was performed on an Axiovision M2 fitted with an Apotome optical sectioning unit and Axioview software.

Suppression of *mep-1* larval lethality

Suppression of *mep-1* larval lethality was performed as in Cui et al., 2006. When raised at 25°C, *mep-1(q660)* homozygotes arrest as L1-L2 larvae. RNAi plates were seeded with 15-20 synchronized JK2906 [*mep-1(q660)* IV/nT1(qIs51; GFP)] L1 larvae, and plates were shifted from permissive temperature (15°C) to restrictive temperature (25°C). When P_0 worms reached L4 (~2 days), they were transferred to new RNAi plates and allowed to lay embryos. Over the course of 4 days, GFP(+) worms (m*ep-1(q660)* heterozygotes), were removed, and GFP(-) *mep-1* homozygotes were allowed to develop and scored 3 days later according to body size by dissection microscope. Larvae that did not progress in development during these 3 days were considered arrested.

Assay for germline transgene desilencing

The assay for germline silencing was performed using the strain PD7271 (*pha-1(e2123)* III (ts); ccEx7271) as Cui et al., 2006, using a strain carrying a highly repetitive multi-copy array of *let-858*::GFP, composed of >100 copies of pBK48 (Tabara et al., 1999; Kelly & Fire 1997). This array expresses nuclear localized GFP in all somatic cells, but no or reduced GFP in germ cells (Kelly & Fire et al., 1997; Kelly & Fire, 1998). When maintained at >20°C, pha-1(+) gene on the array selects for maintenance of the array.

To perform the assay, synchronized L1 PD7271 worms were seeded unto RNAi plates and incubated to adulthood at 25°C. For genes resulting in maternal effect sterility (*mes-2, mes-3, mes-4, mes-6, mrg-1*), germline desilencing was assayed in the P_0 generation. For the additional RNAi's, P_0 adults were transferred to new RNAi plates,

and the F_1 adults were examined. Fifty to one hundred worms were scored per treatment per experiment. Both arms of the adult gonad were observed, and if GFP expression was present in one gonad arm, the animal was scored as GFP+. Levels were not quantified.

Transgene desilencing assay

GFP expressing strains were also used to examine additional desilencing phenotypes; these strains including both integrated and nonintegrated arrays expressed in both somatic and germline tissues. The properties of these strains are summarized in Table 3.

These assays were carried out similar to the germ line desilencing assay, scoring P_0 and F_1 adults raised at 25^{o}C. GFP was scored on an Axioscope 63x. Images were collected on the same settings, and differences in germ line vs. soma were quantitated when appropriate.

Somatic expression of *let-858*::GFP was quantified using ImageJ. Pixel intensity of 30-100 intestinal nuclei was measured and averaged between different experiments. Different let-858::GFP lines showed no difference in ratio intensity between RNAi and control.

Assay for RNAi defective

The HC57 strain expresses *myo-2*::GFP in the pharyngeal muscle, *myo-3*::GFP in the body wall muscle, and a GFP targeting dsRNA hairpin construct driven by the pharynx specific *myo-2* promoter. This strain was designed to allow for monitoring of both localized and systemic RNAi (Winston et al., 2002). Synchronized L1 worms were

Table 3. Transgenic strains used in desilencing assay

Strain:	Transgenic:	Repeats:	Mode:	Promoter:	Expression:
HC195	nrIs20[sur-5::GFP]	Simple	Integrated	Ub.	Soma
SM439	pxEx68 [sur-5::GFP, pRF4]	Simple	Array	Ub.	Soma
AZ212	unc-119(ed3) ruIs32[unc-119(+) pie-1::GFP::H2B] III	Complex	Integrated	Germline	Germline
PD7271	pha-1(e2123) III; ccEx7271 [pBK481 (let-858::GFP); pha-1(+)]	Simple	Array	Ub.	Ub.
KW1127	ccEx7291[let-858:GFP (pBK481) + pRF4 rol-6]	Complex	Array	Ub.	Ub.
KW1336	unc-4(e120) let-858(cc500) II; Ex1336 [pBK48(let-858::GFP); pRF4]	Complex	Array	Ub.	Ub.
NL2507	pkIs1582[let-858::GFP + rol-6(su1006)]	Complex	Integrated	Ub.	Ub.

plated to RNAi and raised at 20^{o}C. F_1 L1 progeny were transferred to fresh RNAi platesand analyzed in early adulthood using a GFP dissecting scope. When RNAi is defective, GFP increased fluorescence in the pharynx (if localized) and body wall muscle (if systemic).

Statistical analysis

Statistical analysis of *hpl-1; hpl-2* suppression and *mep-1* suppression were performed using logistic regression models, adjusting for variation between experiments. Using this model, the odds of surviving past the L1/L2 stage (i.e., control) was calculated in comparison to controls. In these assays, the regression coefficient reports the log odds of survival. Logistic regression analysis was also performed to estimate the odds of

desilencing for each RNAi treatment compared to controls. The regression coefficient reports the log odds of desilencing. In all analyses, estimates were adjusted for variation between different experiments.

Significance relating to the average number of progeny per worm in the *hpl-1; hpl-2* assay was calculated using the Student's T-Test, assuming homoscedastic variance. The statistical difference between the fluorescence intensity of somatic expression of *let-858*::GFP on empty vector and *T19B4.5* RNAi was calculated using the Chi-Square test. These findings were reported to be significantly different if associated with p-values <0.05.

Clustering analysis of this data was performed using Mathematica's DendrogramPlot[] function. The plot was generated by agglomerative clustering with complete linkage (i.e., farther neighbor) of the first two principal components (based on variance) and represents Euclidean distance of unnormalized data. Data from each experiment were weighted equally for a total of 16 independent variables.

I gratefully acknowledge Tala Fakhouri (PhD, MPH) and Kareem Carr (PhD) for assistance in statistical analysis.

Results: Secondary screens

hpl-1; hpl-2 suppression assay: Larval arrest

To address whether genes within the HPL strong suppressor category function in the Mes/PcG pathway, a series of assays was conducted to examine whether loss of these genes would result in classic Mes/Polycomb phenotypes. Prior to any continued analysis of the 11 additional HPL suppressing RNAi clones, each of the clones, including

Mes/PcG and *mes-4* controls, were sequence verified and the *hpl-1; hpl-2* suppression assay was repeated, and the number of worms developing to each larval stage was quantitated. Control *hlp-1; hpl-2* larval worms, when shifted to restricted temperature, produce F_1 progeny that arrest at the L1/L2 stage. These worms are smaller as adults than wildtype, show defects in somatic gonad development and vulval cell fate specification, and are 100 % sterile (Coustham et al., 2003).

As show in Figure 8, there is a range of suppression resulting from RNAi mediated depletion, with *mes-4* suppressing the larval arrest defect to the greatest extent, with 70% of *hpl-1; hpl-2* worms on *mes-4* RNAi surviving to adulthood. *mrg-1* RNAi resulted in a similarly strong arrest (70% reaching adulthood), while *ZK1127.3* RNAi yielded progeny of which 50% develop to adulthood. *mes-2*, *mes-3* and *mes-6* RNAi resulted in a weaker suppression, with the majority of progeny developing to the L3/L4 stage, rather than adulthood. Additional RNAi's produced a range of phenotypes, with *him-17* and *T05B4.5* having the subtlest suppressive effects, with only 5% and <1% of worms reaching L4 in these treatments. By logistical regression analysis, the odds of surviving past L1/L2 in any of the RNAi treatments is significantly different than control RNAi. The regression coefficients and p-values associated with Figure 8 are found in Table 4, and are listed in order of strength of significance. In no case was the sterility of F_1 progeny rescued by RNAi suppression, and both RNAi treated worms and control plates showed significantly less progeny at 25^{o}C than at permissive temperature.

In this assay, *mes-6* does not appear to track with *mes-2* and *mes-3*. As these factors function as a complex, this was somewhat surprising. However, in this assay,

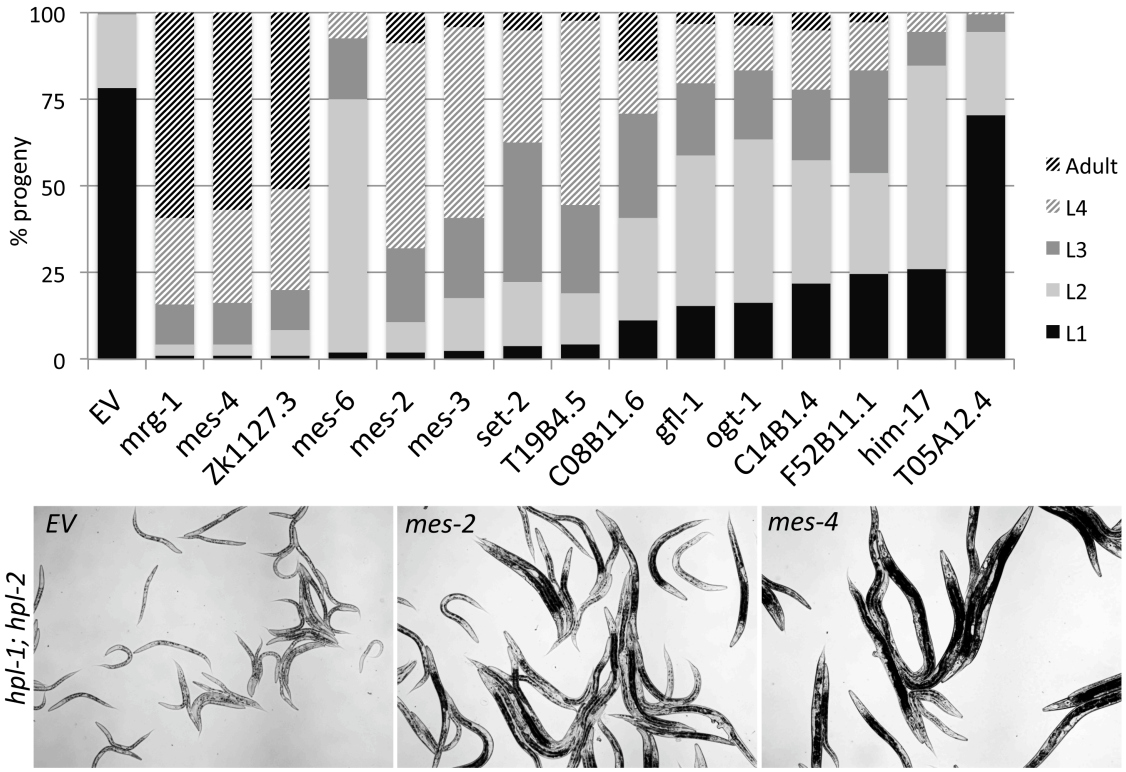


Figure 8. Suppression of *hpl-1; hpl-2* larval arrest. Strength of suppression is graphed in the upper panel. Strength of suppression is related to percentage of progeny developing to late larval stages and adulthood. Following EV (control), RNAi treatments are sorted from strongest to weakest (left to right). The bottom panel shows examples of RNAi treated worm populations at 5x. The majority of EV controls arrest as L1 larvae, while *mes-2* treated worms are able to develop to L4/adult. However, younger larval stages are still observed. The majority of *hpl-1; hpl-2* worms raised on *mes-4* RNAi reach adulthood.

mes-6 RNAi yielded significantly sicker worms compared to *mes-2* and *mes-3*. This is clearly evidenced by the extremely low number of progeny produced by individual worm, as shown in Figure 9. Approximately 40 worms were produced by an *hpl-1; hpl-2* worm grown on empty vector RNAi, a dramatic reduction from the number of progeny produced by wildtype worms, as well as *hpl-1; hpl-2* worms grown at permissive temperature (~200, data not shown). *mes-2* and *mes-3* RNAi treated *hpl-1;*

Table 4. Statistical analysis of *hpl-1; hpl-2* suppression assay

RNAi	Regression Coefficient	Confidence Interval	Pval
mrg-1	8.302	7.578 - 9.025	<0.001
mes-4	8.183	7.538 - 8.827	<0.001
ZK1127.3	7.96	7.333 - 8.587	<0.001
mes-2	7.206	6.576 - 7.837	<0.001
set-2	6.401	5.794 - 7.008	<0.001
mes-3	6.319	5.694 - 6.943	<0.001
T19B4.5	6.305	5.705 - 6.905	<0.001
C08B11.6	5.662	5.062 - 6.263	<0.001
wrd-5	5.567	4.961 - 6.172	<0.001
F52B11.1	5.357	4.741 - 5.972	<0.001
gfl-1	5.304	4.681 - 5.927	<0.001
mes-6	5.021	4.326 - 5.717	<0.001
ogt-1	4.215	3.606 - 4.824	<0.001
T05A12.4	3.283	2.667 - 3.899	<0.001
him-17	3.106	2.456 - 3.755	<0.001

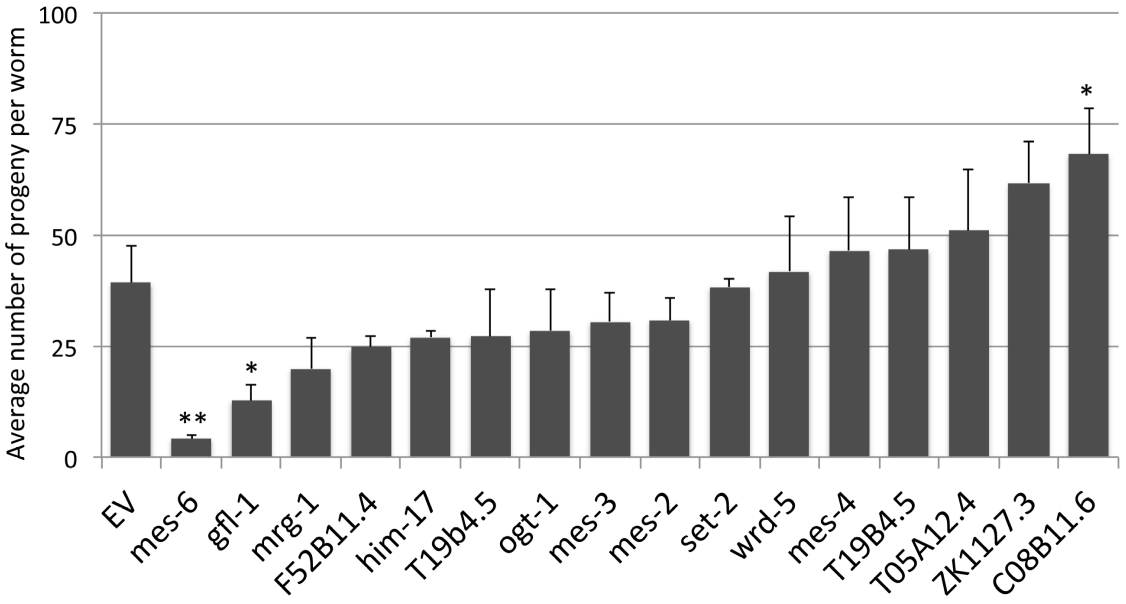


Figure 9. Average number of progeny per worm in *hpl-1; hpl-2* suppression assay. *mes-6* and *gfl-1* show reduced progeny compared to controls, while *C08B11.6* rescues reduced fecundity associated with *hpl-1; hpl-2*. * = pval < 0.05, ** = pval < 0.02

hpl-2 hermaphrodites produced a similar number of progeny to controls (~30/worm), while the average mes-6 RNAi treated worm produced only 4 F_1 progeny. Several reasons might account for this, including strength of RNAi or perdurance of different proteins following RNAi treatment. Alternatively, MES-6 may have additional functions outside of the MES/PcG complex. This difference in number of progeny produced appears to be specific for *hpl-1; hpl-2* mutants, supporting the latter, and further suggesting that this additional function may rely on functional HP1.

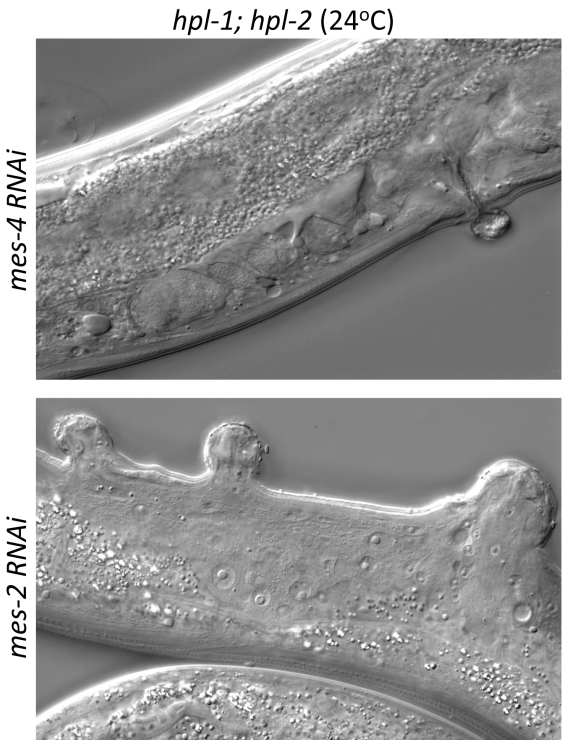
gfl-1 RNAi was also found to result in significantly lower numbers of progeny in the *hpl-1; hpl-2* suppression assay. However, decreased fecundity was also observed in other strains, and is not likely to reflect interaction with HP1 (data not shown). In contrast to the decreased number of progeny produced on *mes-6* and *gfl-1,* some RNAi's appear to rescue the reduced fertility observed in *hpl-1; hpl-2* mutants raised at high temperature. Loss of the SWR1/NuA4 component C08B11.6 was found to significantly increase the number of progeny produced by *hpl-1; hpl-2*mutants, in addition to suppressing the larval arrest of the F_1 generation. Although not significant, the RNAi targeting ZK1127.3 (also a SWR1/NuA4 component) appeared to have a similar effect. The finding that C08B11.6 and ZK1127.3 supress both larval arrest and reduced fertility associated with *hpl-1; hpl-2* might suggest that these proteins might interact with HP1 somewhat differently than other factors.

Suppression of multivulva

In quantifying the *hpl-1; hpl-2* suppression assay, we observed differences in the extent to which the multivulva (Muv) phenotype was suppressed. Ras signaling in the

VPCs is antagonized by two genetically redundant groups of genes, the class A and class B SynMuv genes (Ferguson and Horvitz, 1989). Animals mutant for a gene of either class have a normal vulva, but in animals doubly mutant in both a class A and a class B gene, VPCs ectopically adopt a vulval fate, leading to the multiple vulval (Muv) phenotype. The Muv phenotype results from the failure to downregulate Ras signaling in the vulval precursor cells. At restrictive temperature, *hpl-1;hpl-2* worms also show severe defects in the somatic gonad, including the absence of gonad arms, and complete sterility (Schott et al., 2006). Morphogenesis of the vulval occurs during the L4-adult molt (Sternberg, 2005). Thus, it is not possible to consistently score vulval defects in worms developed only to the L2 – L4 stage. To get around this, we took advantage of the temperature sensitive nature of the *hpl-1; hpl-2* strain. When raised at the semipermissive temperature of 24^{o}C, the majority of F_1 progeny will develop to adulthood, where vulval defects could be reliably assayed.

In this assay, all *hpl-1; hpl-2* suppressors were consistently more developed than empty vector controls, consistent with the suppression phenotypes noted previously. After 8 days, a proportion of empty vector animals remain in larval stages of development, although given even time, all will reach adulthood. At this time point, all progeny raised on suppressor RNAi's had reached adulthood. As shown in Figure 10, a number of suppressors, including *mes-4*, *mrg-1*, *ZK1127.3*, *C08B11.6*, *gfl-1*, and *F52B11.1* fully suppress the multivulva phenotype, with nearly 100% of worms having no evidence of additional vulval protrusions. *wrd-5* also suppresses the Muv phenotype of these worms, albeit to a lesser extent (~50% non Muv). The data presented here are in agreement with previously published data regarding *mes-4*, *gfl-1*, and *ZK1127.3*. These



RNAi	Suppress Muv	Restore fertility
EV	-	-
C08B11.6	+	+++
gfl-1	+	+
ZK1127.3	+	+
mes-4	+	-
mrg-1	+	-
F52B11.1	+	-
wrd-5	+	-
mes-2	-	-
mes-3	-	-
mes-6	-	-
set-2	-	-
T19B4.5	-	-
him-17	-	-
ogt-1	-	-
T05A12.4	-	-

Figure 10. A subset of *hpl-1; hpl-2* suppressors also suppress Muv and restore fertility. The left panel shows images of *hpl-1; hpl-2* worms raised on *mes-4* and *mes-2* RNAi; *mes-2*, but not *mes-4*, results in suppression of the multi-vulva (Muv) phenotype. The results of this assay are tabulated in the right panel. RNAi treatments resulting in suppression of Muv are marked with (+). A subset of Muv suppressors also restored fertility. This restoration was scored as complete (+++) or partial (+).

genes were found to suppress the Muv phenotype associated with a number of different SynMuv A/B combinations, including *lin-15AB(n765)*, *lin-35A(n754)*; *lin-8B(n2732)*, and *lin-36A(n766)*; *lin-8B(n2374)* (Wang et al., 2005; Cui et al., 2006). Notably, these genes were found not to suppress the gain of function mutation in *let-60*/Ras, or the loss of function *lin-1*/ETS, demonstrating that these genes are not Ras pathway genes or involved in general suppression of multivulva phenotypes (Cui et al., 2006; Wang et al.,

2005). Although we did not perform these additional assays with the *hpl-1; hpl-2* suppressors identified in this study, we expect they would behave similarly to *mes-4*. Strikingly, three suppressors resulting in suppression of the Muv phenotype also recued the F_1 sterility associated with this strain. This effect was strongest in *C08B11.6* RNAi, which also suppressed the reduced fertility. Nearly 100% of F_1 animals observed contained embryos. Not only are embryos produced, but were able to hatch and develop, although the extent to which was not assayed. Similar, but somewhat weaker restoration of fertility was observed in *gfl-1* and *ZK1127.3*. While *ZK1127.3* RNAi rescued reduced fertility of the P_0 generation at 25^{o}C (Figure 9), *gfl-1* RNAi treated worms were generally sicker; therefore, this result is somewhat suprising, but suggests that these factors might function together in some capacity. *mes-4* and *mrg-1* suppress *hpl-1; hpl-2* associated growth arrest and Muv phenotypes similarly or even stronger than *C08B11.6, ZK1127.3* and *gfl-1*, yet do not restore fertility. Loss of *mes-4* and *mrg-1* results in maternal effect sterility, this may account for the failure of these genes to suppress sterility, and further suggests that MRG-1 and MES-4 may be required for additional cellular functions. *wrd-5* and *F52Bll.6* are weaker suppressors in the previous *hpl-1; hpl-2* suppression assay, and do not result in restoration of fertility, suggesting these factors may function differently. These findings are intriguing, as the homology of these proteins to yeast counterparts suggest that MRG-1, ZK1127.3, GFL-1 and C08B11.6 are orthologous to members of the SWR1 and NuA4 complexes, which incorporate the histone variant H2A.Z and acetylate histones H2A, H2A.Z, and H4 respectively. In yeast, these function as two distinct complexes, while in *Drosophila* and mammals, homologous factors may function in a common complex known as Tip60/p400, or the closely related SRCAP.

These complexes have not been studied in great detail in *C.elegans*, and it is currently unknown whether these factors function in one or more complexes and mediate chromatin dynamics. In addition, interaction of these complexes with MES-4 have not been previously demonstrated, although based on these initial assays, these factors appear more Mes-4-like than MES-2/MES-3/MES-6 like.

Based on these data, it appears that the SWR1/NuA4 complex(es) antagonize the role of *hpl-2*/SynMuv in control of fertility. As F52B11.1 and WRD-5, which homology predict to function in the H3K4me/COMPASS complex, fail to suppress sterility, it is clear that this is not a general reflection of Muv suppression. MRG-1 is also predicted to function in the SWR1/NuA4 complex, but does not result in rescue of sterility. This may be due to maternal effect sterility of *mrg-1* (Fujita et al., 2002), and suggests that MRG-1 may have additional functions outside this complex.

mes-2, mes-6, mes-6, set-2, T19B4.5, *him-17*, *ogt-1*, and *T05A12.4* do not suppress the Muv defect, suggesting that i) suppression of *hpl-1; hpl-2* is not a result of interaction with the SynMuv pathway, and ii) this group of suppression is functionally distinct from *mes-4*, *mrg-1*, *C08B11.6*, *gfl-1, F52B11.1* and *wrd-5*. In accordance with the findings from our analysis of *hpl-1; hpl-2* at semipermissive temperature, previous screens performed to identify SynMuv suppressors failed to identify these factors (Cui et al., 2006). *mes-2/mes-3/mes-6* have previously been associated with allele specific suppression of the Muv phenotype of *lin-15AB(n765e)*; however, this gene is encoded by the X chromosome, and suppression in this instance can be explained by the role of Mes/PRC2 in X chromosome dosage compensation (Cui et al., 2006; Garvin et al., 1998).

Maternal effect sterile and germ line morphology

The strongest phenotype associated with the MES factors is the maternal effect sterility for which they are named. *mes* mutant gonads have also been shown to contain a reduced number of germ cell nuclei and abnormal cellular morphology, including enlargement of the nucleus and "coagulated looking" cytoplasm. We analyzed the *hpl-1; hpl-2* suppressor mutants for sterility, maternal effect sterility, and germ line morphological defects in both N2 (wildtype) and worms carrying an integrated array expressing histone H2B::GFP under the control of the germ line-specific *pie-1* promoter (Capowski et al., 1991; Paulsen et al., 1995; Garvin et al., 1999).

With the exception of *mrg-1*, which has previously been associated with the *mes* phenotype (Fujita et al., 2003), RNAi mediated knockdown of the other 10 identified suppressors failed to show sterility defects, maternal or otherwise. While none of the RNAi's induce complete sterility, some mild germ line defects are observed in a low percentage of worms (less than 20% in all cases). For example, *him-17* resulted in germ line organization defects, likely associated with its role in meiotic prophase (Reddy & Villeneuve, 2004). *gfl-1*, *F52B11.1,* and *T19B4.5* also show a small percentage of germ lines that appear disorganized, lacking oocytes, or containing endomitotic oocytes, which appear to contain multiple nuclei. This is consistent with *gfl-1* having previously been associated with embryonic lethality (Dudley et al., 2004). *gfl-1* also produced a low number of progeny in the *hpl-1; hpl-2* suppression assays, suggesting this RNAi may result in a generally sick phenotype. *F52B11.1* and *T19B4.5* may also function in germ line development. However, as these phenotypes were relatively low and highly variable, these findings were not pursued further.

The fact that the *hpl-1; hpl-2* suppressors identified by this study do not cause strict maternal effect sterility or more global germ line deformation phenotypes is not entirely surprising. Multiple laboratories, including the Ahringer and Vidal labs, have conducted genomewide RNAi screens that would have included sterility and/or reduced fertility within the screened phenotypes. Other screens (i.e., Simmer et al., 2003) used hypersensitive RNAi strains to perform genomewide screens. In these assays, *T19B4.5* was identified as a maternal effect sterile (in the *rrf-3* background (Simmer et al., 2003)), supporting our hypothesis that *hpl-1; hpl-2* suppressors function in the Mes pathway, as well observation that *T19B4.5* RNAi with respect to germ line morphology.

These data suggest that although the *hpl-1; hpl-2* suppressors may interact with the Mes/PcG or Mes-4 pathway(s), they are unlikely to required for MES function in the germ line. Instead, these proteins might be required for optimal MES activity, aid in the localization or targeting, function with the Mes pathway during early embryonic cell fate decisions, or antagonize the activity of the MES/PRC2 complex, similar to MES-4. Based on homology, it is unlikely that the *hpl-1; hpl-2* suppressors identified by our screen function as a direct repressors. Current models suggest that MES-4 activates the expression of an autosomal repressor that participates in X chromosome silencing (Refer to Figure 3). Perhaps the potential activator complexes (i.e., COMPASS/MLL and/or NuA4/SWR1) contribute to the expression of this repressor. This may account for why depletion of these molecules appears to result in phenotypes more similar to *mes-4* than *mes-2/mes-3/mes-6*.

H3K27me3 staining

We next examined the concentration and localization patterns of Mes/PRC2 catalyzed H3K27me3 marks during embryogenesis. In wildtype animals, H3K27me3 is present in all germ line nuclei and enriched on the X chromosome, consistent with this chromosome being silenced in the germ line (Bender et al., 2004; Reinke et al., 200). H3K27me3 is also present in nuclei of wildtype embryos, and becomes enriched in the germ line precursor cells Z2/3 late in embryogenesis (Bender et al., 2004). In *mes-2/3/6* mutants, however, H3K27me3 is lost from the germ line of the P_0 generation and from all early embryonic cells of the F_1 generation (Bender et al., 2004).

In wildtype embryos, H3K27me3 is fairly uniform, with areas of enrichment, and areas that appear less intensely stained, as shown in Figure 11 (and previously by Bender et al., 2004). H3K9me2 was used as a comarker, and in the early embryos, is more punctate, with the strongest stained areas coincident with but not always overlapping with domains of H3K27me3. As shown in Figure 11 (and previously by Bender et al., 2004), *mes-2*, *mes-3* and *mes-6* RNAi treatment resulted in a dramatic decrease in H3K27me3 levels. RNAi targeting the 11 *hpl-1; hpl-2* suppressors did not affect levels or localization of H3K27me3; an example is shown in Figure 11. Most RNAi treatments had no affect on the H3K9me2 comarker, although *him-17* resulted some decrease in H3K9me2 staining in very early embryos, which may be consistent with a role for HIM-17 in mediating H3K9me2 in the germ line (Reddy & Villeneuve, 2004; Maine et al., 2005). MES-2 encodes the SET domain responsible for this methyl mark, and MES-2/3/6 have been shown to form a complex, with interdependent requirements for

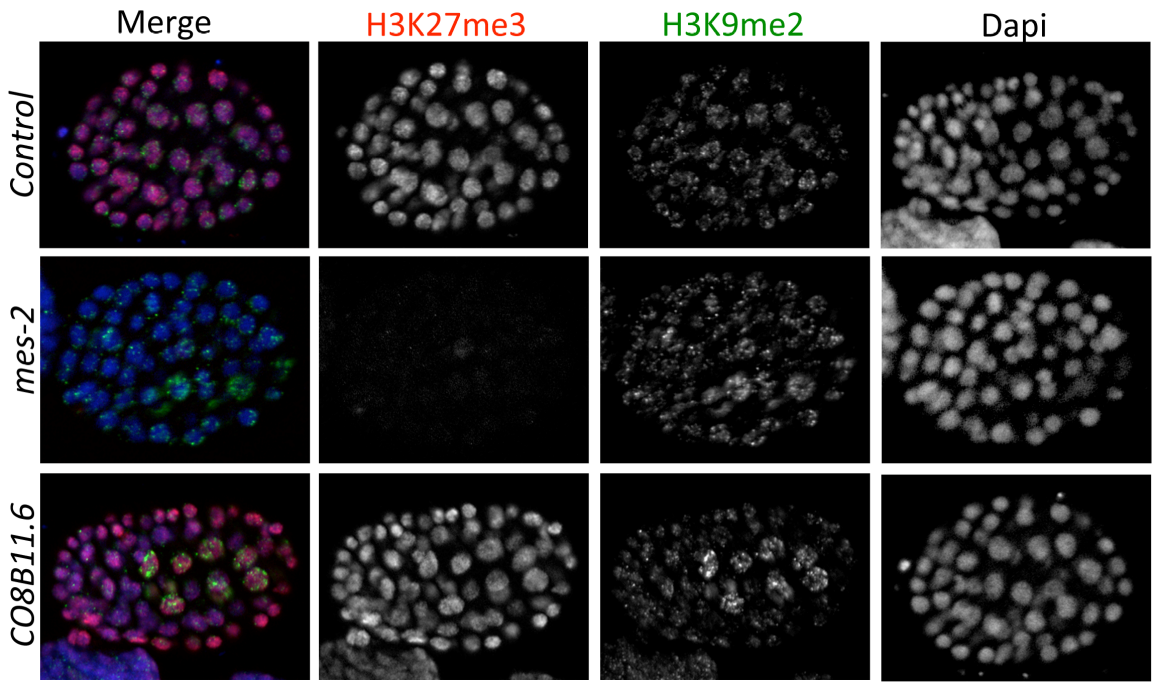


Figure 11. H3K27me3 staining in *mes-2* and *hpl-1; hpl-2* suppressors. 8E stage F1 embryos dissected from parents raised on RNAi. Compared to controls, *mes-2* embryos show greatly reduced H3K27me3 staining. Surprisingly, embryos lacking these marks are able to hatch and grow to adulthood. The *hpl-1; hpl-2* suppressors identified in this study fail to show changes in global H3K27me3 staining; C08B11.6 is shown as an example.

localization to chromatin; unless this complex itself is disrupted, or MES complex targeting is severely abrogated, a dramatic change in H3K27me would be unlikely (Holdeman et al., 1998; Xu et al., 2001). However, it should be noted that the germ line was not analyzed in these experiments; it is possible that staining patterns in *hpl-1; hpl-2* suppressors may affect patterning of H3K27me3 in the mitotic/meiotic phases of the germ cell.

Based on these findings, it is unlikely that the *hpl-1; hpl-2* suppressors identified in this study are required for the MES/PRC2 enzymatic activity. One caveat to these experiments is that antibody staining looks at the embryo from a very global "all or none"

point of view, and subtle changes in H3K27me3 concentration at specific loci would be very difficult to detect. In addition, while levels of H3K27me3 may not significantly change in response to RNAi, it is possible that targeting of MES/PRC2 complex is effected, a defect that could only be detected by ChIP analysis (Whetsine & Strome lab, pers. communication). Alternatively, misregulation at the level of gene expression could also be tested, perhaps analyzing genes known to be affected in *mes-2* mutants (Yuzyuk et al., 2009). We have also considered looking at more subtle phenotypes that might be associated with proteins required to "read" or "interpret" the H3K27 mark, and may function as corepressors, including ectopic enrichment of active marks such as H3K4me or phosphorylated Pol II on the X chromosome prior to the 100 cell stage, or increased in H3K9me2/3 in the PGC. However, analysis of the phenotypes may also present difficulties.

Suppression of *mep-1* larval lethality

The next assay involved suppression of the *mep-1* larval arrest phenotype. The SynMuv factor MEP-1 is a component of the NuRD complex, and is required for suppression of germ line factors in somatic cells (Unhavaithaya et al., 2002). The loss of function *mep-1(q660)* mutant induces L1/L2 arrest when raised at the restrictive temperature (25^{o}C). This arrest is thought to be the result of germ line expression programs becoming active in the somatic tissue due to the lack of suppression by SynMuv components. Larval lethality associated with mep-1 is rescued by depletion of *mes-2*, *mes-3*, *mes-4*, and *mes-6,* as is the ectopic expression of the p-granule marker PGL-1 (Unhavaithaya et al., 2002).

As shown in Figure 12, reduction of the *hpl-1; hpl-2* suppressors induces a range of *mep-1* rescue phenotypes, from strong, with over 75% of worms developing into adults (as in *mes-4* and *mrg-1*), to more intermediate phenotypes (>25% develop to adult), to weaker but significant suppression, in which the majority of worms develop to L3/L4 stages, as opposed to the controls, which arrest as L1/L2. Logistic regression analysis of this data demonstrates that all *hpl-1; hpl-2* suppressors, with the exception of *T05A12.4* are statistically different from controls; these data are listed in Table 5. The regression coefficient reports the odds of surviving past the L1/L2 stage.

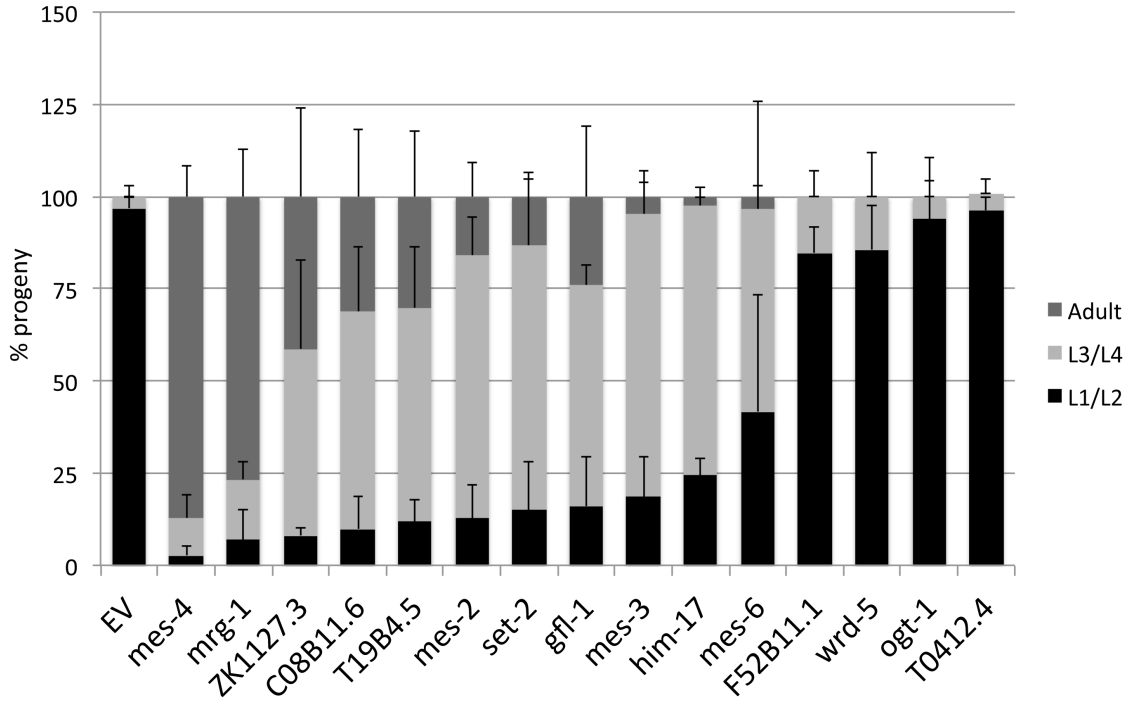


Figure 12. Suppression of *mep-1* by *hpl-1; hpl-2* suppressors. Following the empty vector control, suppressors are ranked in order of strength of *mep-1* suppression.

Table 5. Statistical analysis of *mep-1* suppression assay, ranked in order of significance.

RNAi	Regression Coefficient	Confidence Interval	Pval
mes-4	6.896	5.874 - 7.918	<0.001
mrg-1	6.222	5.433 - 7.012	<0.001
C08B11.6	5.934	5.249 - 6.619	<0.001
ZK1127.3	5.88	5.176 - 6.585	<0.001
T19B4.5	5.529	4.959 - 6.100	<0.001
mes-2	5.41	4.733 - 6.088	<0.001
set-2	5.264	4.633 - 5.895	<0.001
mes-3	5.204	4.599 - 5.810	<0.001
gfl-1	4.902	4.276 - 5.528	<0.001
him-17	4.537	3.963 - 5.110	<0.001
mes-6	3.709	3.158 - 4.260	<0.001
wrd-5	1.915	1.314 - 2.516	<0.001
F52B11.1	1.707	1.039 - 2.376	<0.001
ogt-1	0.938	0.183 - 1.693	0.0149
T05A12.4	0.541	*n.a.*	0.157

Assay for germline transgene desilencing

An additional phenotype associated with MES genes is desilencing of repetitive transgenes in the germ line. The strain PD7271 carries a multicopy extrachromosomal transgene array of a GFP tagged ubiquitously expressed gene, *let-858* (Kelly et al., 1997). Expression of *let-858*::GFP is detectable in all somatic lineages, and is absent in the germ line (Kelly et al., 1997). Although sporadic germline expression is observed at 25^{o}C in empty vector controls (Cui et al., 2006), this assay was performed at high temperature to allow for i) strongest expression of GFP and ii) strongest RNAi phenotypes. We had hoped that genes previously demonstrated to have a role in germline silencing (i.e., *mes-2, mes-3, mes-4, mes-6* (Kelly & Fire, 1998), and *mrg-1*, *F52B11.1* (Cui et al., 2006) would be verified by our examination and thereby validate findings regarding analysis of our other suppressors.

Knockdown of genes that result in maternal effect sterility and a necrotic germ line are nearly impossible to score for germ line desilencing in the M-Z- generation, which lack the majority of germ cell nuclei. Therefore, we assayed these defects in the M+Z- generation (P_0, raised from L1 on RNAi, progeny of these worms are 100% sterile). As shown in Figure 13, *mes-4* and *mrg-1* RNAi showed significantly increased expression of *let-858*::GFP in the germ line relative to controls. Cui and colleagues (who our analysis is based on) found that *mes-4* and *mrg-1* RNAi resulted in 66% and 73% germline GFP respectively. However, these researchers may have scored in the F_1 generation for all treatments, regardless of germ line phenotypes. If our experiments had been quantified in this fashion, similar or even higher levels of expression may have been observed, as the majority of the remaining identifiable germline nuclei were GFP(+) (Senchuk et al., data not shown). We were reluctant to score this as such, however, based on observations that necrotic *C.elegans* cells show increased levels of autofluorescence (Gerstbrein et al., 2005). Additional studies have also demonstrated a role for *mes-2, mes-3 and mes-6* in germ line silencing of transgenic arrays, which by our analyses, was not observed (Kelly & Fire, 1998). This may be due to differences in methods; Kelly & Fire (as well as Takasaki et al., 2007), introduced the repetitive *let-858*::GFP array into different null mutants (*mes-2, mes-3, mes-6, mes-4* and *mrg-1*). *mes-2* (M+Z-) mutants were absent for *let-858*::GFP expression the germ line, as in wildtype, while 85% of *mes-2* (M-Z-) germ lines expressed GFP (Kelly & Fire, 1998). *mes-3* and *mes-6* (M+Z-) germ lines expressed GFP in 56% and 15% of germ lines (respectively), while the M-Z- cohorts were 88% and 100% GFP(+) (Kelly & Fire, 1998). The PRC2 findings are weaker than *mes-4*, which expressed GFP in 100% of germ lines in both the M+Z- and

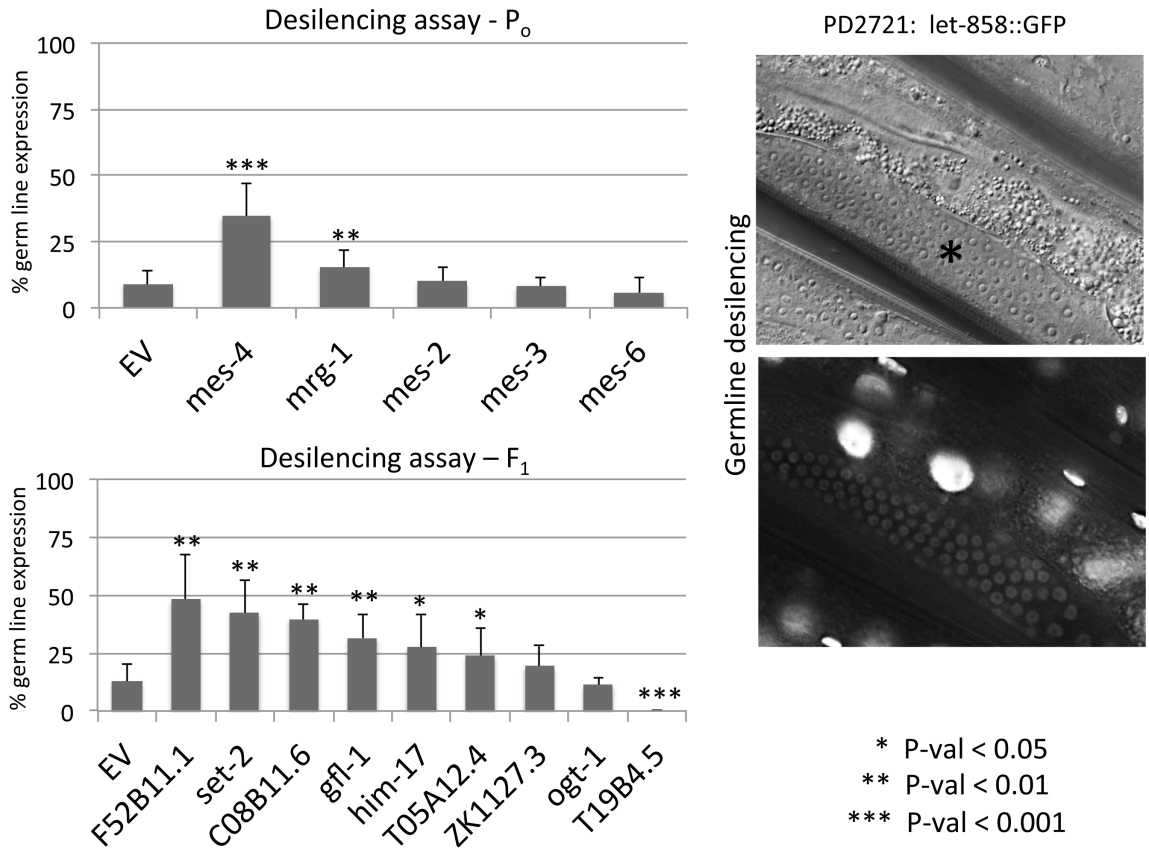


Figure 13. Germline desilencing of the let-858::GFP tandem array. Germline expression of let-858::GFP was scored in the Po generation of RNAi treatments resulting in maternal effect sterility. The remaining *hpl-1; hpl-2* suppressors were scored in the F1 generation. An example of a PD2721 transgenic animal with germline expression of shown. The asterisk marks the germ line. Expression in these nuclei is lower than the surrounding somatic tissue.

M-Z- generations (Kelly & Fire, 1998). Similar to *mes-4*, *mrg-1* (M+Z-) mutants showed nearly 100% germline desilencing (Takasaki et al., 2007). Based on these findings, it is possible that while RNAi targeting *mes-2*, *mes-3*, and *mes-6* was strong enough to induce loss of H3K27me3 in embryos as well as 100% sterility in the next generation, yet not sufficient knockdown to induce desilencing of the repetitive transgene.

Several previous studies have used this assay to examine a number of genes, including a number of *hpl-1; hpl-2* suppressors identified by this study. This assay has

previously been used to analyze the role of additional factors identified by our screen, and comparison of these studies suggest that this assay is, for lack of a better word, finicky. For example, *C08B11.6* and *F52B11.1* RNAi mediated knockdown induced 5% germline GFP expression in F_1 adults (Cui et al., 2006). Our assay found the depletion of these genes induced stronger phenotypes than previously observed, inducing 40% and 48% expression of germline GFP, respectively. Cui and colleagues also examined *gfl-1, wrd-5*, and *ZK1127.3*, and found that these genes did not appear to induce germ line desilencing; these results are in accord with our data. One additional study, which assayed F_2 adults found that *ZK1127.3* did induce germ line desilencing (Wang et al., 2005); this finding contrasts with our work and that of Cui et al. This study also found *gfl-1* did not result in germ line expression, although this was observed in our assay (Wang et al., 2005; Senchuk et al., unpublished). In accord with our data, Wang and colleagues found that *T19B4.5* did not induce desilencing. Somewhat surprisingly, based on its role in mediating H3K9me2 in the germline, and the fact that repetitive arrays are heavily modified with this mark, the *let-858*::GFP transgene was found not to be de-repressed in *him-17* mutants, although we found that *him-17* RNAi was associated with a small, but significant increase in germline expression (Bessler et al., 2007; Senchuk et al., unpublished). A similar low level of GFP expression was observed following RNAi depletion of *T05A12.4*, while depletion of the O-GlcNAc transferase (*ogt-1*) has no effect on germline repression of the transgenic array.

Our analysis also demonstrated that SET-2 is required germ line repression of transgenic arrays, which has not previously been characterized, but fits well with the hypothesized role of SET-2 in the MES/PcG pathway. Depletion of *set-2* leads to 42% of

F_1 adult germ lines expressing GFP in one or both gonads. The mechanism responsible for this desilencing is unknown. As other *hpl-1; hpl-2* suppressors, as well as HPL-2 itself, are chromatin associated, it is likely these affects are exerted at the level of heterochromatin regulation (Couteau et al., 2002). As several RNAi components, including DCR-1, MUT-16, MUT-7 and DRH-1 also demonstrate this phenotype, it is possible that heterochromatin formation in this context may rely on an RNA based mechanism (Kim et al., 2005).

Assay for general desilencing

As shown in Figure 13, it was noted that RNAi targeting *T19B4.5* consistently resulted in fewer GFP (+) germ lines when compared to controls. We found this particularly striking as the somatic expression of *let-858*::GFP was consistently 2.5 (+/- 0.5) fold brighter in *T19B4.5* compared to controls, as shown in Figure 14. Due to the large size, intensity of intestinal nuclei were measured, but changes in intensity were observed throughout the somatic tissue, including body wall muscle, pharynx, hypodermal, and neuronal cell types (data not shown).

We initially hypothesized that somatic desilencing might be due to misregulation of the repetitive array, and that *T19B4.5* might be a soma specific corollary of the MES factors. We tested this hypothesis using a simple tandem array of ubiquitous marker *sur-5*::GFP (Acetoacetyl coenzyme A synthetase) (Gu et al., 1998; Yochem et al., 1998). If *T19B4.5* is required for silencing of repetitive arrays in the soma, we would also expect to see increased expression from the *sur-5*::GFP array. To address whether the observed desilencing was due to unpaired status of the array in the germline, we tested a similar

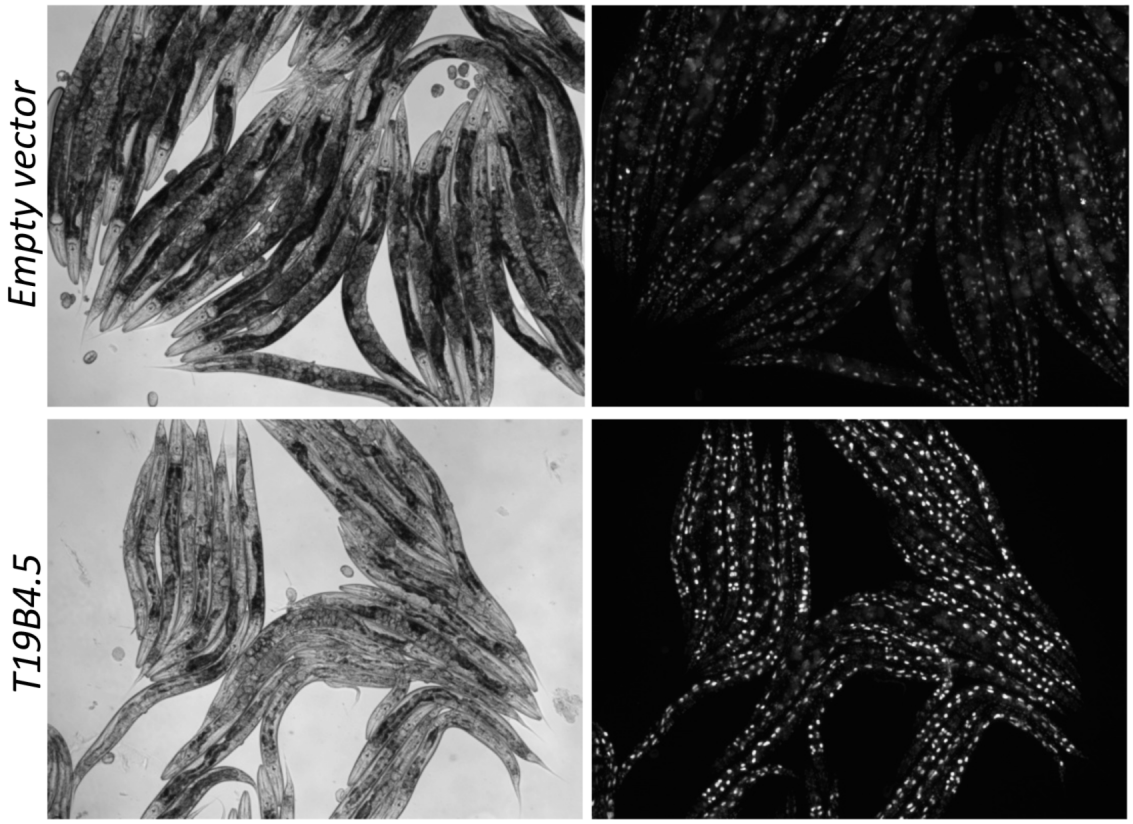


Figure 14. Increased somatic expression of let-858::GFP in T19B4.5. Compared to empty vector controls, *T19B4.5* RNAi induces a 2.5 fold increase in somatic expression in the F1, but not Po generation. T19B4.5 worms are also pale, sickly and slow growing.

sur-5::GFP construct that had been integrated into the genome by irradiation. However, neither of these strains showed increased GFP expression on *T19B4.5* RNAi.

We next compared transgenic strains carrying different forms of the *let-858*::GFP array, including KW1127 and KW1336, which contain complex extrachromosomal transgenic arrays composed of the same *let-858*::GFP reporter interspersed with a large number of random *C.elegans* genomic fragments, as well as NL2507, in which a similar array was integrated into the genome (Kelly et al., 1997). GFP expression in these strains is comparable to the PD7271 strain (simple array), although these strains show expression of *let-858*::GFP in germ cell nuclei (Tabara et al., 1999, Senchuk et al., data

not shown). It did not appear that these changes in *let-858* reporter expression had any significant phenotypic consequence, as might be expected from such dramatic overexpression.

Surprisingly, RNAi mediated knockdown of *T19B4.5* in each of these strains still increased somatic fluorescence, despite the complex, and in the case of NL2507, integrated nature of these arrays. Increased somatic expression did not occur in the P_0 generation, suggesting that passage of the array through the germ line may be essential (Senchuk et al., data not shown). In addition, increased expression was not observed until the L3 stage of F_1 development. A similar pattern was observed in the F_2 generation: expression in embryo-L2 was equivalent to controls, with increased expression starting at the L3 stage and growing more pronounced in L4 and adults (data not shown). This stage specificity suggests that this may be a regulated process rather than a general desilencing.

Even more striking was the observations that in each of the *let-858*::GFP lines, in which the complex nature of the transgenic element allow for germ line expression, the germ line appears to be more strongly suppressed following depletion of *T19B4.5*. This observation is depicted in Figure 15. The data shown in Figures 14 and 15 suggest that T19B4.5 is involved in the positive regulation of germ line gene expression in addition to the suppression of somatic expression. However, changes in GFP expression were not observed in the germline specific *pie-1*::GFP::H2B (AZ212), the HC57 RNAi sensor strain, carrying *myo-2*::GFP and *myo-3*::GFP, or in any of the *sur-5*::GFP constructs tested.

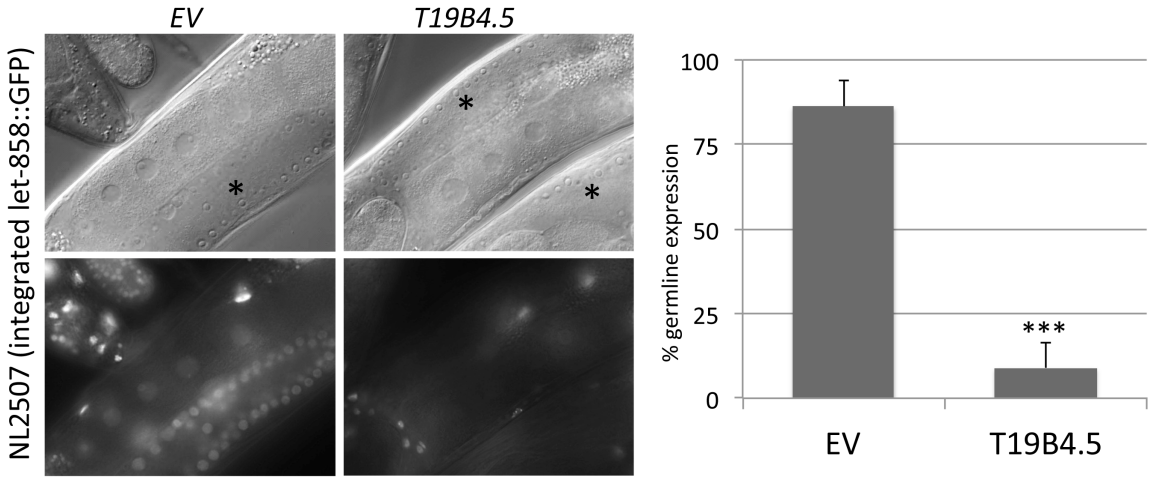


Figure 15. Germline repression of complex, integrated let-858::GFP construct by T19B4.5. F1 worms raised on T19B4.5 RNAi show a dramatic reduction in germline expression of let-858::GFP in this and other strains tested. Asterisks mark the germ line (two worms are visible in the *T19B4.5* image). P-value = < 1E-5

One interesting hypothesis that may account for these observations suggests that *T19B4.5* is involved in regulation of germline competent expression rather than germline specific expression. Previous studies have demonstrated that high copy arrays are differentially modified in the germline, with somatic specific arrays are marked with H3K9me3, while germ line competent arrays are marked with H3K9me2 (Bessler et al., 2010). Perhaps in the absence of *T19B4.5*, marks associated with germ line expression are misread, and interpreted as somatic expression, leading to the observed absence of germline expression but increased somatic expression.

Based on this hypothesis, *sur-5*::GFP may not have been an ideal candidate to determine whether the changes in transgenic expression are due to array composition (i.e., repetitive or complex), array localization (extrachromosomal vs. integrated), and/or promoter competence, as it seems that *sur-5*::GFP expression may be a soma-specific (Gu et al., 1998; Yochem et al., 1998). Whether this is due to the gene itself, or the

composition of various SUR-5 transgenic constructs is unclear. In addition, SUR-5 appears to play some role in vulval development and Ras signaling (Gu et al., 1998). As Mes/PcG pathways interact so closely with the SynMuv pathways, the findings regarding this strain might have proved difficult to interpret.

An alternative hypothesis is the *T19B4.5* may more directly regulate a subset of genes, including *let-858*. This may represent an interesting finding, as *let-858*, which encodes the highly conserved nucampholin, shares some similarity to eukaryotic initiation factor eIF-4 gamma, is nuclear localized throughout development and is at least partially associated with chromatin (Kelly & Fire, 1998). *T19B4.5* is required for germ line proliferation, early embryogenesis and tissue differentiation (Kelly & Fire, 1998). Based on these phenotypes, it's possible that LET-858 might play a role in MES/PcG mediated gene silencing, heterochromatin formation, and/or cell fate commitment during development. However, we were unable to identify a role for *let-858* in our screen due to a severe RNAi phenotype in all mutant backgrounds tested.

One mechanism by which T19B4.5 may mediate specific repressive events might through interaction with SET-18 (based on the Vidal lab interactome database). SET-18 is an uncharacterized protein containing a SMYD domain (SET and MYND (Zinc finger), often involved in transcriptional repression, ubiquitin ligase and/or histone methyltransferase activity (Gottlieb et al., 2002; Matthews et al., 2009).

Assay for RNAi defective

This assay was performed using a strain expressing *myo-2*::GFP in the pharyngeal muscle, *myo-3*::GFP in the body wall muscle, and a dsRNA hairpin targeting GFP driven

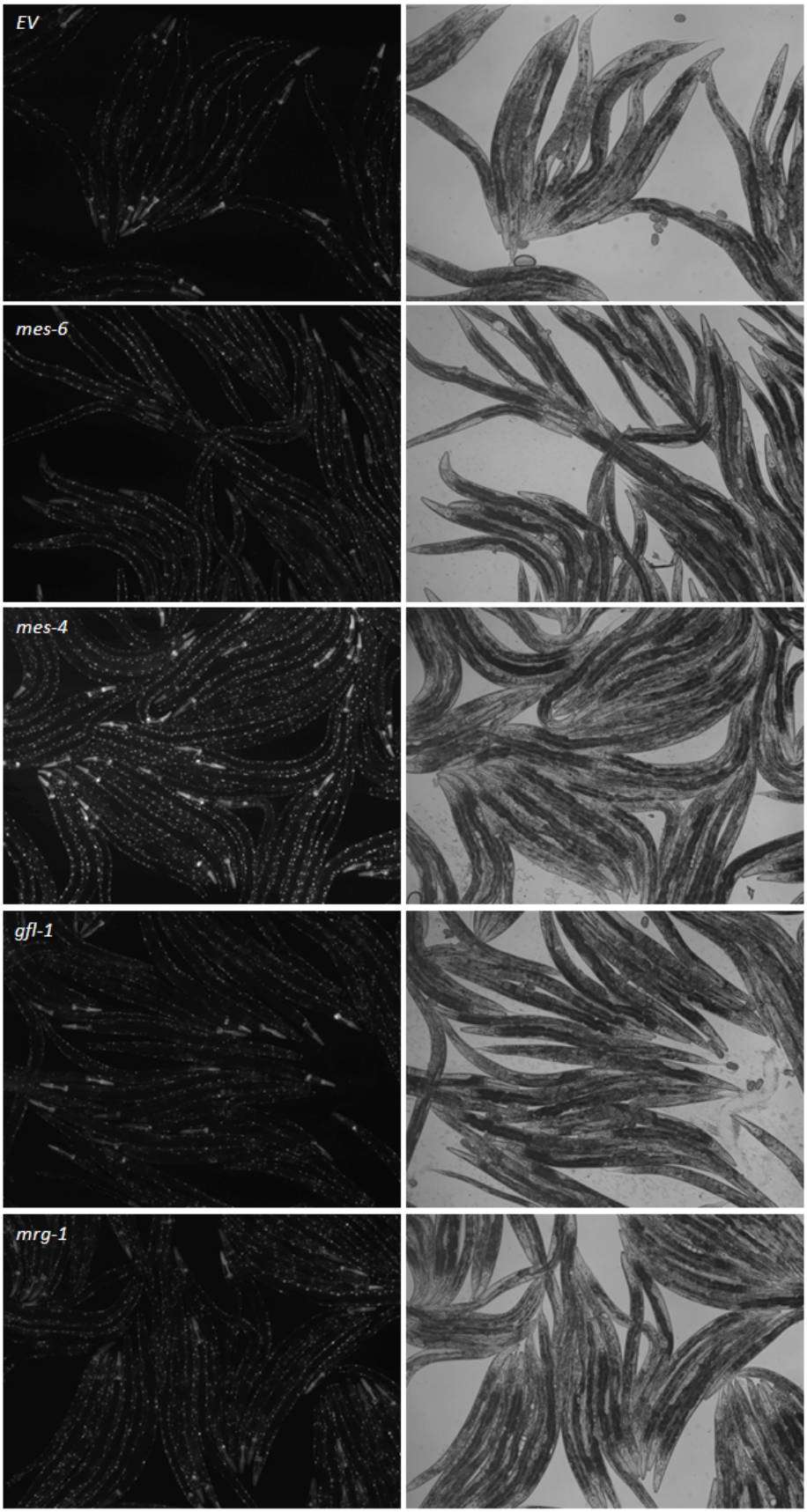
by the *myo-2* promoter. The use of this strain to examine systemic and localized RNAi effects was previously described (Winston et al., 2002). Based on the ascribed role of the Mes/PcG genes in RNAi, and the growing appreciation for the RNA based silencing mechanisms in gene silencing and heterochromatin formation, we assayed whether any of the 11 *hpl-1; hpl-2* suppressors identified in this research function in the classical RNAi pathway. *mes-4* clearly displayed the strongest phenotype in this assay (see Figure 16). P_0 worms on *mes-4* RNAi showed a dramatic upregulation of GFP in both the body wall muscle and the pharynx, indicative of defects in both systemic and localized RNAi. This finding is consistent with previous reports demonstrating the role of MES-4 in regulation of RNAi (Lehner et al., 2006; Dudley et al., 2002; Kim et al., 2005). RNAi targeting *mes-2, mes-3*, and *mes-6* also lead to changes in GFP expression in the RNAi sensor strain. These RNAi's induce an interesting phenotype, in which pharyngeal GFP appears decreased relative to controls, suggesting that depletion of *mes-2, mes-3*, and *mes-6*, leads in increased efficacy of localized RNAi. In these same worms, GFP expression in the body wall muscle was increased, indicating that systemic RNAi requires the Mes/PRC2 pathway. Previous studies have suggested a role for Mes/PcG genes and MES-4 in RNAi, by coinjection and suppression of a lethal dsRNA (*mom-2*) (Dudley et al., 2002).

This analysis demonstrated a stronger phenotype in *mes-4*, but could not address the possibility that PRC2 and MES-4 might function differently in RNA dependent silencing, perhaps by regulating different steps in the pathway, or PTGS vs. TGS. MES-4 is required for RNAi, while MES-2/MES-3/MES-6 appear to negatively regulate localized RNAi, but be required for systemic RNAi. We know from previous studies that

the role of MES/PRC2 in RNAi is dependent on levels of dsRNA (Tabara et al., 1999). Perhaps this complex plays some role in regulation of dsRNA concentrations and/or processing by RdRPs during endogenous gene silencing. It is possible that local and systemic RNAi respond differently to RNA triggers, and in this way resemble PTGS and TGS, respectively. If RNAi machinery normally dedicated to induction of systemic RNAi is not activated, as in the absence of *mes-2/mes-3/mes-6*, this may account for enhancement of local RNAi, as RNAi machinery is focused on the task at hand. For instance, MES-2/MES-3/MES-6 might be involved in directing a pool of dsRNA to RdRPs that are required to induce cell nonautonomous production. Alternatively, loss of these factors may have some affect on transgene stability.

Depletion of the additional suppressors demonstrated that a subset of these factors also appear to function in the RNAi pathway(s). These include MRG-1, F52B11.1, WRD-5, GFL-1, HIM-17, TI9B4.5, SET-2 and T05A12.4. Knockdown of these genes leads to an intermediate phenotype, with increased expression of GFP in body wall muscle and a weak increase in pharyngeal expression, as shown in Figure 16. Some of these data are in agreement with previous analyses; for instance, GFL-1 has been shown to play a positive role in RNAi (Dudley et al., 2002; Kim et al., 2005; Cui et al., 2006). However, our analysis found that MRG-1 functions in the positive regulation of RNAi, although Cui and colleagues (2006) found that MRG-1 not to be required. MRG-1 was also identified in a screen for factors required for the persistence of RNAi over multiple generations (Vasthenow et al., 2006), which supports our findings. Our data also support a role for F52B11.1, which contradicts a previous analysis (Cui et al., 2006). Cui and colleagues also claim that WRD-5 is not an RNAi associated factor, which is in contrast

Figure 16. Phenotypes associated with RNAi sensor strain. The RNAi sensor strain contains myo-2::GFP (pharynx), myo-3::GFP (bodywall muscle), and a dsRNA hairpin targeting GFP driven by the myo-2 promoter. In this assay, MES factors show strikingly different phenotypes. *mes-4* induces desilencing of GFP in the body wall muscle and the pharynx, as expected of a factor required for RNAi-mediated knockdown. *mes-2, mes-3,* and *mes-6* (shown) result in increased GFP in the body wall muscle, with decreased GFP expression in the pharynx. RNAi targeting many of the *hpl-1; hpl-2* suppressors resulted in increased body wall expression. Image on page 134.


EV
mes-6
mes-4
gfl-1
mrg-1

with our findings, as well as an additional study (Simonet et al., 2007). Other studies demonstrated RNAi functionality for T19B4.5 (Kim et al., 2005), ZK1127.3 (Kim et al., 2005; Cui et al., 2006), C08B11.6 (Cui et al., 2006), and SET-2 (Simonet et al., 2007), in accordance with our data. Together, these data suggest that many of the *hpl-1; hpl-2* suppressors identified by our study may function in the RNAi silencing pathway.

There are a number of reasons data from different studies might conflict. First, a number of different assays were used in these different studies, including injection and feeding, and previous studies have shown there are fundamental differences in the specific RNAi response depending on the concentration of trigger RNAi and on the way the dsRNA is administered. For example, *ZK1127.3*, one of the suppressors identified in our study, was also found to suppress phenotypes associated with feeding RNAi, but not injection (Kim et al., 2005). Concentration of dsRNA is also a factor; *mes* genes have been shown to respond differently based on the concentration of trigger RNAi, and appear to be bypassed completely when injection concentrations are low, leading to induction of the targeted RNAi phenotype (Tabara et al., 1999). RNAi efficacy is also scored in differently in the various analyses, including lethality, visible phenotypes (i.e., Dpy), and RNAi sensor strains. As we know that these factors can also influence transgenic expression, our data (collected using an RNAi sensor strain), as well as others (Cui et al., 2006; Kim et al., 2005), must be interpreted carefully.

Discussion: Two classes of MES interacting factors

Of the 738 RNAi clones tested, 236 showed enhancement/suppression of one or more heterochromatin mutants and do not appear associated with general enhancement

(i.e., enhance all background mutations). Strikingly, all known members of the Polycomb/Mes pathway were found to strongly suppress the *hpl-1; hpl-2* early larval arrest phenotype. 11 additional genes showed a similar phenotype – strong suppression of *hpl-1; hpl-2*, but failure to effect *met-2* or *mes-3*. We hypothesized that this signature might be indicative of factors functioning in the Mes/PcG repressive pathway.

To determine whether other *hpl-1;hpl-2* suppressors represent factors functioning in the Polycomb pathway, a number of secondary screens were performed, including tests of maternal effect sterility (*mes*) and germ line morphology, histone methylation status, rescue of *mep-1* larval arrest, germ line transgene desilencing, and a role in RNAi; all phenotypes associated with *mes* loss of function.

Although RNAi knockdown of *mes-2/mes-3/mes-6* and *mes-4* suppress *hpl-1; hpl-2* larval lethality, Figure 8 shows that *mes-4* RNAi allows a greater percentage of animals to survive past L4 (57% of progeny survive to adulthood in *mes-4*, compared to 9% adult development in *mes-2*). As shown in Figure 10, *mes-4* also suppressed the Muv phenotype of *hpl-1; hpl-2* adults raised at semipermissive temperature, completely restoring normal vulval development, which *mes-2*, *mes-3*, and *mes-6* did not. In our assays, *mes-4* also more strongly suppressed the larval arrest phenotype of *mep-1* homozygous mutants, with 87% of *mep-1* mutants raised on *mes-4* RNAi able to reach adulthood, compared to 16% in *mes-2* (Figure 12).

It has previously been shown that *mes-4* phenotypes are distinct from those associated with *mes-2/mes-3/mes-6* loss of function. For instance, *mes-4* null mutant hermaphrodites gonads contain fewer germ nuclei compared to other *mes* mutants (Capowski et al., 1991, Garvin et al., 1998). MES-4 also appears to play a more

pronounced role in RNAi (Wang et al., 2005; Cui et al., 2006) and functions in the repression of PolII in the PGCs, a role which appears to be independent of MES-2 function (Furuhashi et al., 2010). In addition, MES-2/PRC2 is essential in the regulation of gene expression during the transition from plasticity to cell fate commitment, with loss of *mes-2* inducing prolonged pluripotency and expression of 2E and 4E genes, as well as a failure to upregulate 8E/differentiation genes (Yuzyuk et al., 2009). Based on the Nuclear Spot assay, changes in gene expression may result from failure to compact chromatin in *mes-2* mutants (Yuzyuk et al., 2009). It is currently unknown whether MES-4 plays a role during this transition. Based on the previously observed differences in function and phenotypes, we reasoned that it is unlikely that the different effects observed between *mes-4* and *mes-2/mes-3/mes-6* is our assays is likely attributable to differences in RNAi efficacy.

We noted that a number of *hpl-1; hpl-2* suppressors identified in this work phenocopy *mes-4* in these assays: Strong suppression of *hpl-1; hpl-2* larval lethality, restoration of normal vulval development, and rescue of *mep-1* larval arrest. We hypothesize that these genes interact more closely with MES-4 than with MES-2/PRC2. This cohort of genes might i) function with MES-4 to repress X-linked gene expression, or ii) function in other MES-4 dependent pathways; these models are not mutually exclusive. We also predict that *hpl-1; hpl-2* suppressors with secondary assay phenotypes more similar to *mes-2/mes-3/mes-6* are likely to interact more directly with PRC2 than MES-4, and may play a role X-chromosome silencing and/or the transition from plastic to committed.

Genes with "*mes-4-like*" phenotypes include *MRG-1* and *ZK1127.3*, proteins previously shown to interact by yeast two hybrid (Li et al., 2004). MRG-1 is homologous to fly MSL3, yeast Eaf3 and human MRG15, proteins found associated with a number of HAT containing complexes, including NuA4/Tip60, as well as the counteractive HDACs and histone demethylase complexes (Carrozza et al., 2005; Joshi & Struhl, 2005; Keogh et al., 2005; Morillon et al., 2005; Martin et al., 2006; Taverna et al., 2006; Hayakawa et al., 2007; Larschan et al., 2007; Moshkin et al., 2009). By homology, ZK1127.3 is related to yeast Eaf7 and fly MrgBP, chromatin associated proteins also thought to function in the NuA4 complex. Additional proteins that may share a *mes-4-like hpl-1; hpl-2* suppression phenotype include *gfl-1* (GAS41) and *C08B11.6* (Arp6); homologs of these proteins also function in chromatin modifying complexes including NuA4 and SWR1 (Lu et al., 2009). In addition, members of the COMPASS complex, including *F52B11.1* and *wrd-5* appear *mes-4-like* in some, but not all assays. In contrast, the phenotypes associated *mes-2* and *mes-3* correlate well with the phenotypes observed following loss of SET-2 and T19B4.5. The phenotypes of *him-17*, *ogt-1*, and *T05A12.4* are often weaker than *mes-2* and *mes-3*, indicating that the interactions between these factors are perhaps more indirect. Data from all assays were combined, weighted identically, and subjected to agglomerative cluster analysis, shown in Figure 17. This plot nicely summarizes our findings and strikingly resembles the interactions predicted by homology.

The remaining sections focus on the *hpl-1; hpl-2* suppressors identified in our primary screen and how these factors might function in the MES/PcG pathway. Proposed models and future experiments designed to address these models are discussed.

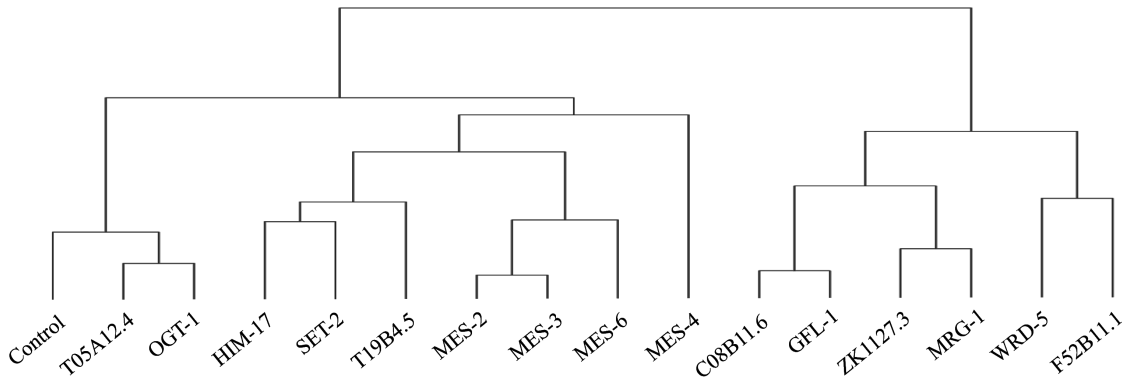


Figure 17. Cluster analysis of *hpl-1; hpl-2* suppressors. Agglomerative clustering of all phenotypes associated with secondary analyses described in this chapter. T05A12.4 and OGT-1 are more similar to control than other *hpl-1; hpl-2* suppressors, suggesting that these factors may not function in the Mes/PcG pathway. HIM-17 and T19B4.5, along with the previously identified PcG interacting factor SET-2 cluster with MES-2, MES-3, and MES-6. MES-4 is somewhat of an outlier, but still localized in this cluster, consistent with previously identified roles in antagonism of MES/PRC2 function. MES-4 is also closely related with the third cluster, containing components of the NuA4/SWR1 and COMPASS components.

Mes-4-like suppressors

As shown in Figure 3, there are currently two models of MES/PRC2 complex antagonism by MES-4. The first model predicts that MES-4/H3K36me activates the expression of an autosomally encoded repressor that localizes to the X chromosome and inhibits transcription. This repressor may or may not interact with H3K27me3, but as this mark is MES-4 independent, it is unlikely that the MES-4 associated repressor recruits H3K27me3. An alternative model suggests that MES-4/H3K36me3 repels a repressor, focusing the repressive function on the X chromosome. As the main function associated with Mes-4-like *hpl-1; hpl-2* suppressors is transcriptional activation, the simplest model to account for function of these complexes in the Mes pathway suggests

that these complexes are involved in activating the expression of the X-localized repressor molecule, rather than the repressor molecule itself. Previous ChIP studies in M+Z- *mes-4* germ lines only identified only four upregulated autosomal genes (Bender et al., 2006), and as such, the repressor targeted for activation by MES-4 is this model is unknown, therefore, testing this hypothesis is currently out of reach.

COMPASS/MLL

The mixed lineage leukemia (MLL) protein and its Drosophila homolog, Trithorax, exist in COMPASS (complex of proteins associated with Set1) like complexes and mediate H3K4 methylation and gene activation (Tenney & Shilatifard, 2005). The enzymatic activity of SET1/MLL family members is thought to be regulated though interaction with a number of additional proteins, including Swd3/WDR5, Swd1/RbBP5, Bre2/Ash2, Spp1/CFP1, and Sdc1/hDPY30 (Tenney & Shilatifard, 2005). Components of the COMPASS complex are highly conserved from yeast to humans, although different sets of the subunits are distributed among different H3K4 HMT complexes in multicellular organisms (Patel et al., 2009; Patel et al., 2011; Steward et al., 2006; Dehe et al., 2006). In many species, COMPASS related gene activation antagonizes PcG repression, preventing inappropriate gene silencing (Tenney & Shilatifard, 2005). This work demonstrates that multiple COMPASS like complexes are likely to function in *C.elegans,* supporting recent findings by the Kelly, Palladino, and Strome labs (Xiao et al., 2011; Li and Kelly, 2011). At least one of COMPASS like complex containing SET-2 functions in the MES pathway, and another complex, composed of a number of shared subunits (WRD-5 and F52B11.1), but lacking SET-2 likely functions more closely with

MES-4 and/or during vulval differentiation. As *wrd-5* and F52B11.1 have subtle, albeit significant, effects on *mep-1* suppression, this second complex may have a less substantial contribution to promotion of germ line cell fates.

SET-2 was identified as an enhancer of the MES sterility in a screen for performed in germ line compromised *mes* M+Z- mutants (Xu & Strome, 2001). We and others have shown that only a subset of COMPASS components, including *set-2*, *wrd-5* and *F52B11.1* enhance *hpl-1; hpl-2* larval lethality (Simonet et al., 2007; Senchuk et al., unpublished). Like *set-2*, *wrd-5* has a mortal germ line phenotype, supporting that these factors function in germ line development, perhaps by interacting with MES pathway (Xiao et al., 2011, Li & Kelly, 2011). Conversely, RNAi of *Y17G7B.2/Ash2*, *set-16/MLL* and *F21H12.1/CFP1* failed to suppress and/or enhanced *hpl-1; hpl-2* larval lethality (Simonet et al., 2007; Senchuk et al., unpublished). Several recent findings support the hypothesis that these components form at least partially independent complexes. First, *set-2; ash-2* double mutants show additive defects in fertility (Xiao et al., 2011). These components may also primarily contribute to different H3K4me marks; SET-2 is required for both H3K4me2 and H3K4me3, but Y17G7B.2/ASH-2 is only required for the H3K4me2, and is not required for H3K4me3 (Xiao et al., 2011). These marks also display different patterns of chromatin localization, with H3K4me3 enriched around the transcription start sites of actively transcribed genes, whereas H3K4me2 displays a broader distribution (Xiao et a, 2011).

SET-2 mediates the majority of H3K4me3 in most developmental stages and the germ line, while ASH-2 contributes to H3K4me3 in embryos, but functions independently of SET-2 to mediate H3K4me2 (Xiao et al., 2011). In fact,

immunostaining of *ash-2* mutants demonstrated that H3K4me2 is higher in the PGCs compared to wildtype embryos, suggesting that ASH-2 might antagonize SET-2 in these cells (Xiao et al., 2011). While this may reflect a defect in the conversion of H3K4me2 to H3K4me3, this interpretation seems unlikely as H3K4me3 is also low in wild-type PGCs (Schaner et al., 2003). Alternatively, antagonism of SET-2 might be accomplished by association of the ASH-2 complex with a histone demethylase, as has been observed in yeast and *Drosophila* (Secombe et al., 2007; Roguev et al., 2003). Interestingly, the *spr-5/LSD1* demethylase, specific for H3K4me, enhances the *hpl-1; hpl-2* phenotype, lending support to this hypothesis, as does the finding that defects associated with *spr-5* defects include germline mortality (Senchuk et al., unpublished; Katz et al,2009).

WRD-5 and F52B11.1, but not SET-2, suppress the Muv phenotypes associated with *lin-15AB* and *hpl-1; hpl-2* (Simonet et al., 2007; Senchuk et al., unpublished). It is possible that WRD-5 and F52B11.1 function as part of a SET-2 independent complex (that may or may not possess HMT activity) that functions in vulval development. Studies have shown that maintenance of H3K4me2/3 is dependent on WRD-5, but independent of AMA-1/PolII (Li & Kelly, 2011). Perhaps this maintenance function is involved in vulval development. An alternative hypothesis suggests that H3K4me2 might play a role in vulval development. While all COMPASS components contribute to H3K4me3 (of which SET-2 mediates the vast majority), western blot and immunofluorescence analyses demonstrated that WRD-5 and F52B11.1 are also required for H3K4me2, which does not appear to require SET-2 (Simonet et al., 2007; Li & Kelly, 2011; Xiao et al., 2011). It's possible that H3K4me2 mediated by these factors contributes to SynMuv interactions, as *wrd-5* and *F52B11.1*, but not SET-2, suppress the

Muv phenotype. As the other COMPASS components did not suppress *hpl-1; hpl-2* larval lethality, a role for these subunits can not be ruled out. Intriguingly, components of a COMPASS/MLL-like complex including SET-16, WRD-5, ASH-2 was pulled out of genomewide screen based on the ability to attenuate Ras signaling, the pathway controlled by SynMuv factors (Fisher et al., 2010). Components of the NuA4 complex also demonstrated this functionality (Fisher et al., 2010). How MES-4 functions in the SynMuv pathway of vulval development is unknown.

Based on these data, the regulation of H3K4 methylation in *C.elegans*, as in mammals, may involve distinct HMT complexes to yield a variety of different outcomes in different tissues and developmental stages. At least one COMPASS like complex is likely involved in the MES pathway, and another, possibly sharing a number of subunits, is involved in vulval cell differentiation. This hypotheses is consistent with the findings demonstrating the functionality of COMPASS/MLL like complexes which lack subunits previously thought the be essential for function and/or stability, including the HMT SET1 (Patel et al., 2009; Patel et al., 2011; Steward et al., 2006; Dehe et al., 2006)

How might the COMPASS like complex interact in the MES pathway? One intriguing hypothesis suggests that similar to its Trithorax counterparts, the SET-2/WRD-5 complex might mark specific targets, including differentiation specific genes, with H3K4me3. If these targets are cooccupied by H3K27me3, the formation of "bivalent domains" might be required to ensure the correct temporal gene expression profiles during development. One might use ChIP analysis to examine whether H3K4me3 marks coincide with H3K27me3 at target promoters developmentally regulated by MES-2/PRC2 (as identified by Yuzyuk et al., 2009). It would also be of interest to use ChIP-

based methods to identify additional protein factors bound to these regions, aiding in the identification of factors required to target COMPASS and/or PRC2, or to identify factors that read one or both of these marks. It is also possible that a switch in COMPASS subunit composition might drive the transition from poised to activated, as has been observed for SWI/SNF like BAF transition from proliferating neural stem/progenitors to postmitotic differentiated neurons (Lessard et al., 2007). Recently, it has also been suggested that histone demethylases might function within the COMPASS/MLL complex (Fisher et al., 2010). While levels of H3K4me and H3K27me appear to be independently regulated at the level of western blot, the demethylase UTX-1 was shown to phenocopy SET-16 COMPASS/MLL components in the vulval tissues, and *utx-1* depletion induced elevated levels of H3K27me3. Unfortunately, *utx-1* RNAi was not in the original screen, but tracking of a demethylase might represent a means of PRC2 regulation. For example, PRC2 might methylate all available H3K27 moieties, and the COMPASS complex "prunes" repressive marks from loci requiring activation. Perhaps this would explain the lack of specific PRC2 specific DNA targeting in *C.elegans* and other species.

Several alternative hypotheses might also account for interactions between MES and COMPASS. First, components of MLL/COMPASS are found associated with the DCC complex involved in repression of the X chromosomes during embryonic development (Pferdehirt et al., 2011). As a major function of the MES pathway involves silencing the X chromosome, this heterochromatin domain would represent a clear candidate where the MES and COMPASS like complexes might interact. However, as *dpy-30* (DCC and COMPASS), as well as other DCC factors (i.e., *sdc-1*) were not identified as strong suppressors of *hpl-1; hpl-2* larval arrest in the Nuclear/RNAi

sublibrary screen (Primary screen, this manuscript) or a Meiosis/Repair sublibrary screen (Senchuk et al., data not shown), this hypothesis seems unlikely, although this interaction has not been definitively ruled out.

An interesting third alternative suggests that MES and COMPASS interactions may involve epigenetic regulation. Epigenetics refers to nongenetic, loci fixed chromatin components capable of influencing transcriptional programs that are inherited and propagated through cell division and development (Campos & Reinburg, 2009). In many eukaryotes, the most common and well-studied epigenetic mark involves methylation of DNA at CpG islands; however, *C.elegans* lack DNA methylation, allowing for a closer examination of other forms of epigenetic marking. Specific histone tail posttranslational modifications may also represent epigenetic marks; however, it is unclear how these marks are maintained, and what differentiates a nonepigenetic histone mark (i.e., those directly regulating transcriptional programs cell autonomously) from a mark that needs to be maintained to guide transcription in subsequent generations.

MES-4 is thought to function as a maintenance HMT, and in the embryo, MES-4 mediated H3K36me marks genes expressed in the maternal germ line (Furuhashi et al., 2011). Recent studies also suggest that H3K4 methylation may play an epigenetic role, evidenced by WRD-5 dependent, but transcription independent marks in early embryos, and by an uncharacterized mechanism, transgenerational extension of life span (Li & Kelly, 2010; Greer et al., 2011). H3K4me marks are also found in the absence of active transcription in zebrafish embryos, suggesting these epigenetic phenomena are likely to be conserved in other species as well (Vasthenow et al., 2010). In the early embryo, it is possible that early transcriptional profiles are established by H3K36me, H3K4me, and

possibly H3K27me. Additional studies by our lab and others will likely address these hypotheses.

SWR1/NuA4/TIP60, H2A.Z

The NuA4 complex is an evolutionarily conserved multisubunit HAT complex, which is recruited to target promoters and participates in acetylation of H4, H2A, and H2A.Z, and is typically thought to induce transcriptional activation. The SWR1 complex is an ATP dependent chromatin remodeling complex, involved in the incorporation of the histone variant H2A.Z, which is important for gene regulation and genomic stability. The incorporation of H2A.Z is thought to change nucleosome dynamics, but the effects on gene expression remain controversial (Marques et al., 2009). In yeast, at least four components are shared between these two complexes. Moreover, recruitment of SWR1 requires NuA4, and evolutionary evidence suggests that overlap may be greater in complex metazoans (Lu et al., 2009). In fact, in mammals and *Drosophila*, the associated factors, including Tip60-p400 and SRCAP, function as a single complex, using different subunit compositions to fine tune complex functionality (Lu et al., 2009; Marques et al., 2009).

Tip60 possesses both histone acetyltransferase and chromatin remodeling activities and can act either as a positive or negative regulator of transcription (Ikura et al., 2000, Cai et al., 2003; Xiao et al., 2003). Tip60-p400 transcriptional regulation seems to be mediated, at least in part, by the incorporation of the histone variant H2A.Z into nucleosomes and by the catalysis of histone acetylation at target genes (Sapountzi et al., 2006, Squatrito et al., 2006). Similar to its potentially dual roles in transcriptional

regulation, Tip60 plays seemingly opposing roles with respect to DNA double strand breaks (DSB). This complex participates in the activation of DSB formation, and has also been shown to be recruited DSB sites where it contributes to DSB repair (Squatrito et al., 2006).

Intriguingly, the Tip60 complex was identified in a large scale RNAi screen for chromatin remodeling proteins involved in ES cell function, and was shown to repress developmental genes in this cell population (Fazzio et al., 2008). Loss of TIP60 function led to defects in self renewal and differentiation capacity, although the expression of pluripotency associated transcription factors (i.e., Oct4) was unaffected (Fazzio et al., 2008). ChIP analyses in mESCs indicate that Tip60 colocalizes with Nanog and H3K4me; these marks are required for Tip60 recruitment. The authors also show that Tip60 induces H4 acetylation at both activated and repressed target genes (Fazzio et al., 2008). Given the role of Polycomb and MES factors in pluripotency, this link is particularly interesting, and suggests that Tip60 components might play a role in the transition from pluripotency to cell fate commitment in *C.elegans*. Based on the observed recruitment to H3K4me, COMPASS/MLL may also be linked to this function.

By homology to the yeast SWR1 and NuA4, a number of components are likely to function in the *C.elegans* NuA4/SWR1 or Tip60 like complex(es). Of these, four were found to suppress *hpl-1; hpl-2*, including *gfl-1* (AF9-like), *mrg-1* (EAF3-like), *ZK1127.3* (EAF7-like), and *C08B11.6* (Arp6 like). Although it did not come up as a positive in the primary screen, the histone variant H2A.Z has been shown by our group and other to suppress *hpl-1; hpl-2* as well as the SynMuv factor *lin-15AB*, suggesting that loss of H2A.Z is also *mes-4-like* (Senchuk et al., unpublished; Cui et al., 2006). Additional

factors, including C17E4.6, CD4.7, SSL-1, and MYS-4 were not among the genes targeted by RNAi in the initial sublibrary. Although not screened, we would hypothesize that these factors might also strongly suppress *hpl-1; hpl-2*. Alternatively, they might behave similarly to *ekl-4*, *trr-1*, *mys-1*, *epc-1*, which were found to enhance all mutant background phenotypes (*hpl-1; hpl-2*; *met-2* and *mes-3*). This enhancement phenotype is likely related to previously annotated RNAi phenotypes, but also suggests that these factors play additional roles during development.

Based on these findings, it is likely that a NuA4/SWR1/Tip60 like complex(es) in *C.elegans* interact with MES-4. Alternatively, some components (including MRG-1, ZK1127.3, GFL-1, and C08B11.6) may form one or more subcomplexes that mediate specific, MES-4 related functions. Data from previous studies support the latter hypothesis, as *trr-1*, *mys-1*, and *epc-1* are SynMuv C genes that repress ectopic expression of *lag-2* in the intestine (Poulin et al., 2005; Ceol & Horvitz, 2004). Conversely, our study and others identified *mrg-1, ZK1127.3, gfl-1* and *C08B11.6* as SynMuv suppressors (Senchuk et al., unpublished, Cui et al., 2006). Cui and colleagues also found that these factors are required for the ectopic expression of *lag-2* observed in SynMuv mutant backgrounds (Cui et al., 2006; Senchuk et al., unpublished). Based on these data, different NuA4/SWR1 like complexes may have opposing functions, with the GFL-1, C08B11.6, MRG-1 and ZK1127.3 complex functioning to promoting gene expression (based on the *lag-2*::phenotype).

Several interesting hypotheses might account for the interaction of NuA4/Tip60 and the Mes/PcG pathway, specifically focusing on the HAT activity (SWR1/Tip60 function, and the incorporation of HTZ-1/H2A.Z is discussed in the following section).

As suggested in the first section, one attractive model suggests that the NuA4/Tip60 complex functions in conjunction with MES-4 to activate an autosomal repressor of the PRC2 complex, thereby preventing association of the PRC2 complex with autosomes and concentrating the repressive activity on the X chromosome. It is also possible that NuA4/Tip60, independently of MES-4, activates an X localized factor that recruits PRC2, which in turn repels MES-4. Alternatively, this complex might activate the expression of a molecule required for targeting of MES-4 to the autosomes, or that autosomal or histone acetylation (or incorporation of H2A.Z) directly recruits MES-4 and/or repels PRC2.

Studies have shown that loss of PRC2 activity results in a global increase in H3K27 acetylation, a mark thought to be antagonistic to PcG mediated silencing (Tie et al., 2009; Pasini et al., 2010). Although NuA4 has not been previously associated with this mark, Mes/PRC2 might be localized to the X chromosome by autosomal H3K27ac, added directly by NuA4 or indirectly mediated by this complex. Alternatively, PRC2 mediated H3K27me3 marks may repel H3K27ac, which would then be restricted to autosomes and might contribute to the localization of MES-4. In addition, previous studies have demonstrated that PRC2 complexes associate with HDACs (Kuzmichev et al., 2002; Pasini et al., 2010; Tie et al., 2003; van der Vlaag & Otte, 1999), it is also possible that targeting a MES/PRC2-HDAC complex to the X chromosome might remove marks of activation in this domain and/or allow methylation of H3K27me3, while MES-4 may be recruited to acetylation marks remaining on autosomes. While, based on the findings of this study, the HAT complex NuA4 would be an excellent candidate to perform this function, we were unable to identify an HDAC like complex that might be

involved with the MES/PcG pathway in this capacity, although MRG-1/Rdp3S has not been ruled out. In the initial screen, however, we identified the histone deacetylase SIR-2.1, which has previously been associated with heterochromatin, as strong *hpl-1; hpl-2* suppressor, and was not analyzed in this work as loss of *sir-2.1* also enhanced *mes-3* sterility.

In *S.cerevisiae* and *Drosophila*, H3K36 methylation has been reported to recruit an HDAC containing Rpd3S complex, resulting in deacetylation of the 3' end of actively transcribed genes (Carrozza et al., 2005; Joshi and Struhl, 2005; Keogh et al., 2005, Bell et al., 2007). In contrast to Set2/dHypb, the loss of which resulted in reduction of H3K36me2/3 and increased histone acetylation, knockdown of dMes-4 had the opposite effect, with reduced H3K9me as well as global reduction in acetylation of histone H4K16 (Bell et al., 2007). Therefore, while Set2/dHypb (and by correlation *C.elegans* MET-1), may actively recruit HDACs and/or interfere with the acetylation of H4K16, MES-4 might have the inverse effect and recruit HAT activity. Although this model is untested in *C.elegans*, one candidate for this might be the NuA4/SWR1 complex.

H2A.Z

SWR1 and the orthologous complexes Tip60/p400 and SRCAP remodel nucleosomes and incorporate the histone variant H2A.Z/*htz-1*, which occupies specific chromatin domains. H2A.Z is a highly conserved and accounts for ~5-10% of all H2A in a nucleus (Marques et al., 2010). Although not required for yeast viability, loss of H2A.Z in other organisms, including *Drosophila*, worms, and mice, results in embryonic lethality (Marques et al., 2010). In *S.pombe*, loss of H2A.Z leads to defects in

transcriptional activation and alterations in transcriptional silencing. In this and other species, the function of H2A.Z remains unclear.

Studies have shown that H2A.Z is found at promoters and regulatory regions of active genes, including insulators and enhancers, as well as areas of facultative and constitutive heterochromatin, but is rarely found within the gene bodies of actively transcribed genes (Hardy & Robert, 2010). H2A.Z is often incorporated into nucleosomes flanking the nucleosome free regions associated with transcriptional start sites, and is thought to promote nucleosome instability (Marques et al., 2010). However, it has also been hypothesized that the increased mobility of H2A.Z containing nucleosomes might also negatively impact transcription by blocking key regulatory sequences. In human, *Drosophila* and *C.elegans*, genomewide studies have shown that H2A.Z is often associated with RNA PolII, and may play some role in PolII pausing and/or recruitment (Hardy & Robert, 2010; Marques et al., 2010; Whittle et al., 2008). In addition to the promoters of actively transcribed genes, H2A.Z also occupies large domains occupying several hundred kilobases, and can also regulate the localization of chromatin to the nuclear periphery, a domain generally associated with transcriptional inactivity (Marques et al., 2010; Brickner et al., 2007). In *C.elegans*, HTZ-1 is found at significantly lower levels on X chromosomes relative to autosomes (Whittle et al., 2008).

Genomewide analysis of ES cells has demonstrated that the occupancy of H2A.Z and PcG protein of promoters are highly correlated and interdependent (Creyghton et al., 2008). By RNAi, the authors show that incorporation of H2A.Z during mouse ESC differentiation is essential for proper lineage commitment (Creyghton et al., 2008). Moreover, following differentiation, H2A.Z occupies a different subset of genes,

suggesting that this relocalization may contribute to the dynamic changes in gene expression during the transition from pluripotency to cell fate committed (Creyghton et al., 2008), a role that would be highly similar to the Mes/PRC2 complex in *C.elegans* (Yuzyuk et al., 2009).

Another potential link between the Mes/PcG pathway and SWR1 function comes from studies in flies, where it has been shown that the PRC2 and the PhoRC associated protein Pho/YY1 (which is the only sequence specific DNA binding protein identified as part of the Polycomb family), physically interacts with the INO80/SWI-SNF remodeling complex (Klymenko et al., 2006). As the SWR1 complex is also an INO80 family remodeler, one intriguing model of Polycomb targeting might involve interaction with SWR1/NuA4 complexes.

As studies in yeast have shown that histone acetylation by NuA4 is a prerequisite to incorporation of H2A.Z by the SWR1 complex. Therefore, it is possible that these factors interact in the Mes/PcG pathway based on their requirement for H2A.Z incorporation into the genome. As H2A.Z may function may exert either positive or negative effects on gene expression, many models are possible, including those discussed above. H2A.Z/HTZ-1 might be integrated into autosomes where it might i) directly attract MES-4 and/or repel MES/PRC2, or ii) activate an autosomal encoded repressor of the PRC2 function.

MRG-1

Of all the *hpl-1; hpl-2* suppressors identified by this study, the most well studied in connection with Mes/PcG pathway is MRG-1, an *hpl-1; hpl-2* suppressor with a

striking *mes-4-like* phenotype in all secondary screens analyzed in this study. MRG-1 is a chromodomain binding protein related to human mortality factor related gene 15 (MRG15), and based on knockout mouse phenotypes, is required for cell proliferation and embryo survival (Tominaga et al., 2005). MRG-1 was identified in *C.elegans* based on a maternal effect sterile phenotype resulting from necrotic degradation of germ cells (Fujita et al., 2002). Like MES proteins, MRG-1 is maternally provided, expressed ubiquitously, becomes enriched in germline precursor cells, and is required for silencing of X-linked genes known to be targeted by MES-4 (Fujita et al., 2002; Takasaki et al., 2007).

MRG-1 is similar to MES-4 in its autosomal localization, but MRG-1 is not required for MES-4 localization, and MRG-1 localization is unaffected in *mes-2* and *mes-4* loss of function mutants (Takasaki et al., 2007). It is possible that MRG-1 may be recruited and bind by H3K36me2 via its chromodomain, as has been observed in yeast EAF3 (Carrozza et al., 2005; Keogh et al., 2005). Takasaki and colleagues found that MRG-1 localization was unaffected by loss of MES-4 mediated H3K36me; however, the authors did not address H3K36 methylation induced by MET-1. In yeast, there is only one H3K36 HMT (SET2). It's possible that in *C.elegans*, MRG-1 may interact with both transcription independent (MES-4) and transcription dependent (MET-1) mediated H3K36me, although it may preferentially interact with one form. This might explain why MRG-1 localization was not affected in *mes-4* mutants. A preference for seemingly identical marks might be mediated by 3' vs. 5' enrichment, additional cofactors, and/or di- vs. tri-methylation. MRG-1 binding H3K36me may account for it's autosomal enrichment. Based in the similar phenotypes of *mes-4* and *mrg-1*, we might predict that

MRG-1 might bind and stabilize H3K36me mediated by MES-4. If MRG-1 is able to bind both MET-1 and MES-4 mediated H3K36me, MRG-1 could be involved in the repression of MES/PRC2; MRG-1 binding to MET-1 mediated H3K36me3 in a *mes-4* mutant might be sufficient to maintain exclusion of PRC2, and account for the observation that H3K27me3 patterns are unchanged in the *mes-4* mutant. However, MRG-1 remains associated with autosomes in very early *mes-4* (-) embryos which have been shown to lack H3K36me3, suggesting that MRG-1 in *C.elegans* is not localized by binding H3K36me3 (Takasaki et al., 2007; Rechsteiner et al., 2010). By antibody staining of later stages of embryogenesis, MRG-1 is not required for PGCs to acquire transcriptional competence of the PCGs, and as activated PolII is not preciously detected in P4 precursor cell, it is unlikely that MRG-1 interacts with MES-4 in this capacity (Takasaki et al., 2007).

In addition to functioning in the NuA4/SWR1 complex(es), MRG-1/EAF3 also functions in the Rpd3S HDAC complex, which is recruited to H3K36me2/3 to suppress spurious transcription within actively transcribed genes (Lee & Shilatifard, 2007). Loss of EAF3 function induces global changes in acetylation of the yeast genome, with increased acetylation in coding regions, and reduced acetylation at promoters, consistent with reduced gene expression (Reid et al., 2004). Other components of the Rdp3S complex are homologous to the *C.elegans* proteins HDA-1, HDA-2, HDA-3; none of which were found to strongly suppress *hpl-1; hpl-2*, suggesting that the NuA4/HAT related complex interacts with the Mes/PcG pathways rather than Rdp3S/HDAC.

MES-2/PRC2 like genes

T19B4.5

One of the most striking findings of this analysis was the characterization of the novel gene T19B4.5. By sequence homology, T19B4.5 may be nematode specific, although functional homology is not ruled out. By motif searches, T19B4.5 contains 3 coiled-coil domains, which may be important for interaction with nucleic acids or other proteins. In our analyses, *T19B4.5* was found to suppress *hpl-1; hpl-2* larval arrest and rescue *mep-1* larval lethality better than *mes-2* (30% adults in *T19B4.5* vs. 16% adults in *mes-2*). The most interesting phenotype was loss of *let-858*::GFP expression in the germ line, inducing this repression even in when the arrays were complex in nature and integrated into the genome resulting in consistent expression in the germline. This finding suggests that T19B4.5 may promote germ line expression. As shown in Figure 14, RNAi targeting *T19B4.5* also induced dramatic upregulation of *let-858*::GFP in the soma, irrespective of the type of transgene analyzed. These data suggest that T19B4.5 may function to inhibit repression in the somatic tissue.

What mechanisms might contribute to increased somatic expression in *T19B4.5*? Although our data demonstrate that *let-858*::GFP is the only transgene demonstrating dramatic changes in expression, a previous study showed that loss of *T19B4.5* also induces desilencing of transgenic arrays in the seam cells and nonneuronal cells (Kim et al., 2005). One of these transgenes was also tested in our study: *sur-5*::GFP. One important difference between these two analyses is that the Kim study tested desilencing of transgenes in an RNAi-enhanced background (i.e., *eri-1* and/or *rrf-3*). However, the interpretation of this result is complicated by the fact that T19B4.5 plays a positive role in

RNAi (Kim et al., 2005; Senchuk et al., unpublished), and RNA-based mechanisms are known to be involved in the regulation of transgenes and repetitive elements. ERI-1 and RRF-3 prevent silencing of repetitive transgenes in the soma, likely by repressing RNAi based mechanisms of silencing (Simmer et al., 2002). One might predict that loss of genes promoting RNAi would enhance transgene expression in an *eri-1*/*rrf-3* mutant background, and this appears to be the case for a number of RNAi genes, including *mes-4* and *gfl-1* (Kim et al., 2005). However, these genes do not induce increased somatic expression in an *eri-1/rrf-3* (+) background, as *T19B4.5* appears to, suggesting that T19B4.5 functions differently than known RNAi genes.

Although less well studied, somatic silencing of transgenes appears to occur at the transcriptional level through RNA dependent mechanisms and histone deacetylases (Grishok et al., 2001; Grishok et al., 2005). The somatic desilencing phenotype of *T19B4.5* is opposite that of the SynMuv factor *tam-1* (tandem array modifier), as well as additional SynMuv genes, including *lin-35*, which are involved in context dependent gene silencing in an RNAi dependent manner. In the soma, loss of *tam-1* induces a dramatic reduction in expression of tandem repetitive arrays (Hsieh et al., 1999), and it is thought that transgene silencing in the *tam-1* mutant occurs at the pre-mRNA stage (Grishok et al., 2005). Based on these data, one might predict that T19B4.5 negatively regulates TAM-1, or alternatively, these factors function in parallel to regulate transgenic expression. These different hypotheses could be addressed by assessing transgenic expression in *tam-1; T19B4.5* double knockout. However, *tam-1* does not induce silencing of complex arrays (Hsieh et al., 1999), which *T19B4.5* clearly does in the case of different *let-858*::GFP constructs. Therefore, perhaps T19B4.5 also negatively

regulates factor(s) responsible for expression of complex arrays and/or endogenous loci. An alternative model suggests that T19B4.5 activates a repressor, which might function upstream of TAM-1/SynMuv genes as well as factors responsible for the regulation of complex array expression. We have not yet tested whether *T19B4.5* leads to increased expression of endogenous *let-858* and/or other endogenous loci.

The effect of *T19B4.5* observed in the germ line is opposite of that observed somatic tissues, with reduced transgenic expression in the germ cell nuclei, a phenotype that has not been previously identified. A number of models might account for this phenotype. In the simplest model, T19B4.5 might directly activate transgenic expression. Very little is known about T19B4.5, although motif prediction software suggests that this protein contains a coiled-coil domain that may interact with DNA. Secondly, T19B4.5 might activate some other factor required for array expression (i.e., indirect activation). A third model predicts that T19B4.5 might repress a factor that activates a downstream repressor of array expression.

It is also possible that T19B4.5 does not affect the array, but instead directly regulates a subset of genes, including let-858, which encodes the highly conserved nucampholin, shares some similarity to eukaryotic initiation factor eIF-4 gamma, is nuclear localized throughout development and is at least partially associated with chromatin, and is required for germ line proliferation, early embryogenesis and tissue differentiation (Kelly & Fire, 1998). It is possible that the role of T19B4.5 in the MES pathway is mediated through LET-858 or other factors activated or repressed in response to T19B4.5. As noted in the results section, T19B4.5 might interact with arrays and/or endogenous genes through it's proposed binding factor SET-18/SMYD.

Overall, the finding that T19B4.5 appears to oppose Mes function in the germ line (as a repressor) as well as SynMuv factors in the soma (as an activator), suggests that T19B4.5 may represent an important target of future study. We are currently characterizing phenotypes associated with a null mutation of *T19B4.5*, as well as building expression constructs using the endogenous promoter and UTR to examine the localization patterns of T19B4.5 throughout development. Future studies will also address the affects of loss of T19B4.5 function on germline competent vs. germline specific promoter arrays, as discussed in the results section. We also hope to address the potential role of this factor in transcriptional gene silencing pathways.

O-GlcNAc transferase

N-acetylglucosamine (O-GlcNAc) transferase (OGT-1) catalyzes the addition of O-GlcNAc moieties to nuclear and cytoplasmic proteins at serine and threonine residues. There are several hints from the literature that this modification might interact with the Mes pathway. In mice and *Drosophila*, loss of OGT function is embryonically lethal (Myers et al., 2011; Gambetta et al., 2009). In flies, lethality results from homeotic transformations, with homeotic genes expressed outside their native domains (Gambetta et al., 2009). In *Drosophila*, OGT is encoded by the Polycomb group gene Super Sex Combs (*sxc*), and genomewide profiling has demonstrated enrichment of GlcNAc modifications at Polycomb response elements (PREs) (Gambetta et al., 2009; Sinclair et al., 2009). These GlcNAc bound sites overlap with PhoRC; however, binding of Pho at PREs is unaffected in *sxc* mutants, as are H3K27me levels (Gambetta et al., 2009). These data suggest that OGT enzymatic activity may function downstream of Polycomb

function, perhaps recruited by H3K27me3. OGT also glycosylates RNA PolII, Polyhomeotic, core histones, and various transcription factors, suggesting GlcNAc modification may directly involved in transcriptional regulation (Sinclair et al., 2009; Gambetta et al., 2009). OGT is also an enzymatic component of the human dosage compensation complex (DCC), suggesting that GlcNAc might be involved in higher order chromatin structure of the X chromosome (Love et al., 2010). A recent proteomics based analysis in mESCs demonstrated that GlcNAc modifies a number of proteins involved in maintenance of cell identity, including SOX2 and the Nanog interacting protein ZFP281 (Myers et al., 2011). In these cells, PRC2 is necessary to maintain normal levels of OGT and for the correct cellular distribution of O-GlcNAc (Myers et al., 2011).

Preliminary analysis has shown that localization and levels of OGT and GlcNAc appear unaffected in *mes-2*(-) *C.elegans* embryos (Senchuk et al., unpublished). However, as GlcNAc modification has been associated with a wide array of cellular processes, including signaling, protein turnover, nutrient sensing and gene expression, it is perhaps not surprising that global levels of this modification were unchanged. One interesting future experiment might involve testing this model using ChIP analysis, to address, for example, whether O-GlcNAc modifications are lost on promoters targeted by PRC2 in a *mes-2* mutant. It might also be worth investigating whether this molecule functions during the transition from pluripotency to cell fate commitment.

HIM-17

HIM-17 is a germ line expressed, chromatin localized protein involved in meiotic processes, including double strand break formation (DSB), chiasmata, crossovers, and recombination (Reddy & Villeneuve, 2004). HIM-17, as well as the RdRP EGO-1, are required for correct patterning of H3K9me2 in the meiotic germ line (Reddy & Villeneuve, 2004; Maine et al., 2005). HIM-17 also functions to regulate the balance between germ cell proliferation and meiotic development, inhibiting proliferation and/or promoting meiotic entry (Bessler et al., 2007). However, loss of *him-17* function does not induce desilencing of transgenes in the germ line (Bessler et al., 2007; Senchuk et al., unpublished). HIM-17 has also been shown to genetically interact with the SynMuv protein LIN-35/Rb; codepletion of these factors is synergistic with respect to DSB formation frequency (Reddy & Villeneuve, 2004).

There are two potential models by which HIM-17 might interact with the MES/PcG pathway. First, the stabilization of the H3K9me2 by HIM-17 might recruit MES-2/PRC2 to target loci, or vice versa. This is somewhat unlikely as immunofluorescence assays demonstrate the H3K27me3 and H3K9me2 marks are unaffected in *him-17* embryos. However, this model is not ruled out in the germ line. An alternative model suggests that HIM-17-associated H3K9me2 serves as a template for conversion to H3K9me2 by PRC2. However, Bessler and colleagues suggest that the MES-2 and MET-2 H3K9me marks are acquired independently (Bessler et al., 2010). MET-2 also associates with HIM-17 by MudPIT mass spectrometry analysis (Bessler et al., 2010). However, loss of *met-2* has the opposite effect of *mes-2*/PRC2 and *him-17*, and enhances rather than suppresses *hpl-1; hpl-2* larval arrest (Simonet et al., 2007;

Senchuk et al., unpublished). *met-2* may also weakly enhance *mes-3* sterility (Senchuk et al., unpublished). While these different pathways concerning H3K9me, HP1, and PRC2 clearly interact, the mechanisms of antagonism and/or cooperation remain unclear.

Summary

This dissertation describes a primary screen of >700 nuclear and RNAi associated factors. We identified enhancers and suppressors associated with the three major heterochromatin and transcriptional silencing pathways: *mes-3*/Polycomb, *met-2*/H3K9me2/3, and *hpl-1;hpl-2*/HP1. More than 200 factors were found to suppressor or enhance one of more of these background mutations, but are unlikely to represent general enhancers. We were struck by the observation that all identified members of the Mes/Polycomb pathway strongly suppressed the *hpl-1; hpl-2* larval arrest phenotype, and did not affect *mes-3* or *met-2*. Factors that have been associated with the PRC1 complex did not have this phenotype, suggesting that these genes do not function in a common pathway with MES-2/MES-3/MES-6 (PRC2) and the interacting factors MES-4 (H3K36me) and SET-2 (H3K4me).

A number of additional factors showed the distinctive signature of strong *hpl-1; hpl-2* suppression. These include members of the COMPASS/MLL complex (H3K4me), members of the NuA4 HAT complex, members of SWR1 nucleosome remodeling complex (incorporation of H2A.Z), O-GlcNAc transferase, an enzyme catalyzing the addition of a common and fairly uncharacterized PTM, a novel factor (T19B4.5), and two proteins involved in DNA repair.

To further characterize the potential role of these factors in the Mes/PcG pathway, a number of secondary assays were performed, including suppression of the SynMuv ectopic vulva phenotype, suppression of *mep-1*/NuRD induced larval lethality, germ line desilencing of a repetitive array, and function in the RNAi pathway. Two major findings are associated with these studies. First, factors associated with activating complexes (COMPASS, NuA4/SWR1) demonstrate phenotypes very similar to loss of *mes-4*, suggesting that these complexes may function in cooperation with MES-4, and may contribute to indirect transcriptional repression of the X chromosome. As loss of F52Bl1.1 and WRD-5, but not SET-2, phenocopy *mes-4*, it is likely that the COMPASS complex can function in at least two forms, one of which is SET-2 independent and interacts with the Ras pathway to control vulval formation.

The second finding involves the novel, uncharacterized, and potentially nonconserved T19B4.5. T19B4.5 is associated with a unique phenotype, characterized by overexpression of the *let-858*::GFP transgene in the soma, coupled with strong repression of expression in the germ line. This phenotype is not dependent on the type of array used, suggesting that changes in expression are not related to repetitive elements often associated with transgenic arrays. As this phenotype appears to oppose SynMuv and Mes/PcG function, this gene represents an exciting avenue to pursue in future studies.

APPENDIX A

DEVELOPMENT OF A CONTROLLABLE GENE EXPRESSION SYSTEM

Use of transgenic animals has become a common practice in most *C.elegans* labs. Transgenes are commonly used as convenient comarkers for genetic studies, to study overexpression or ectopic gene expression phenotypes, to study protein localization by expressing fluorescent tagged genes, to assess the role of regulatory elements in expression patterns, to rescue mutant phenotypes, and to perform structure/function analyses.

Several methods have been developed to introduce transgenes into the soma and germline (Merrit & Seydoux, 2010). However, few methods are available to specifically modulate expression of the endogenous gene relative to the transgene, nor to study differential expression from an endogenous locus or a transgenic gene.

Here we describe the use of transgenes from the closely related nematode *C.briggsae*. These transgenes can be introduced into the *C.elegans* genome by standard methods, and can be detected with species specific PCR and modulated by RNAi. We show that *C.briggsae* transgenes are expressed and localize similarly to *C.elegans* counterparts, and can rescue *C.elegans* loss of function and RNAI mediated knockdown

of endogenous gene expression. We also show, by qPCR and western blot, that both mRNA and protein levels are specifically targeted by species specific knockdown.

C.elegans and *C.briggsae* are closely related members of the nematode genus *Caenorhabditis*, and diverged from a common ancestor 30-110 million years ago (Cutter, 2008; Stein et al., 2003). Though morphologically indistinguishable and found in the same ecological niche, these species are more evolutionarily divergent than humans and mice (Stein et al., 2003). We hypothesized that the genetic differences between *C.elegans* and *C.briggsae* might allow researchers to use the *C.briggsae* genome as a tool to engineer transgenes in *C.elegans* lab strains that are can be modulated using speciesspecific RNAi.

Despite the divergence, the gene makeup, gene structure (i.e. exon length and intron placement), and genome assembly of *C.elegans* and *C.briggsae* are highly similar, with substantial synteny despite the observed intrachromosomal arrangements (Stein et al., 2003). Sixty five percent of *C.elegans* genes have a clear *C.briggsae* ortholog, with a mean percent identity of 80% (Stein et al., 2003), similar to the identity observed when human and mouse genomes are compared (Waterston et al., 2002). At the genome level, 52.3% of *C.elegans* sequence aligns to *C.briggsae* (Stein et al., 2003). In addition, almost all noncoding RNAs and operons are conserved between species (Stein et al., 2003). Of the ~20,000 protein coding genes identified in the *C.briggsae* genome sequence, only 800 genes are unique. Moreover, the strongest alignments occur in coding sequence and in the ~1kb upstream of the start site, indicating that promoters are also highly conserved (Stein et al., 2003). It's estimated that 30% of the *C.elegans* genome (~5,700 genes) are essential, meaning the loss of these genes result in zygotic

lethality, maternal effect lethality, and/or sterility (Herman, 1978; Johnsen & Baillie, 1997). These genes likely show above average levels of conservation.

We introduced the *C.briggsae* promoter and coding sequence for two essential *C.elegans* genes by two different methods into the *C.elegan*s genome. *hmr-1*, a classical cadherin, is nearly 60 Kb, and was recombineered into a citirine (a modified GFP) expression vector, injected into the germline and maintained as a complex array under temperature sensitive *cha-1* selection. *C.briggsae* sequence corresponding to the RhoGAP *cyk-4* (2.7 Kb) was used to construct *cyk-4*$^{C.br}$::mCherry and introduced in single copy into the ttTi5605 locus by mosSCI (Frøkjaer-Jensen et al., 2008). Similar to other ortholog pairs, these gene pairs have very similar intron and exon structures, as shown in Figure 18. The unspliced versions of these genes are 51% and 67% identical to their C.*elegans* counterparts, and coding segments share 74-75% identity by global alignment. The *cyk-4* promoter (1.75 Kb) is 54.7% identical to the *C.briggsae* 5' sequence; because of lack of sequencing in the promoter region of *C.briggsae hmr-1*, the promoter regions could not be compared. At the amino acid level, *C.elegans* CYK-4 and HMR-1 share 80-86% identity with the *C.briggsae* equivalents.

We first tested whether the exogenous *C.briggsae* transgenes could rescue when the endogenous gene was reduced by RNAi using constructs available thru Gene Service (Ahringer library clones), which are each one to two kilobases in length. Previous studies demonstrated that duplexes as short as 26 nucleotides can trigger an RNAi response (Parrish et al., 2000). Lack of confirmed *C.briggsae* sequence prohibits full alignments, however, there are two stretches (28 nucleotides and 32 nucleotides) in the

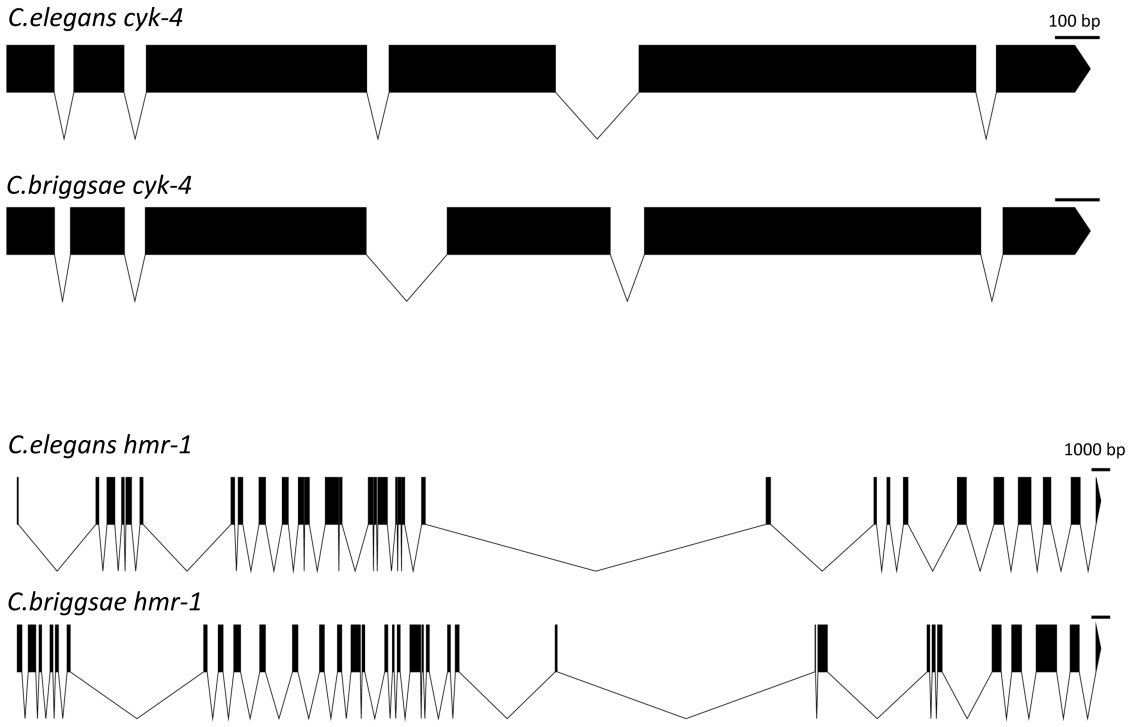


Figure 18. Gene structure of *cyk-4* (Rho GAP) and *hmr-1* (cadherin) in *C.elegans* and *C.briggsae*. Dark boxes represent exons, and introns are represented by lines. Scale bars are indicated.

C.elegans cyk-4 RNAi sequence that would be capable of targeting the *C.briggsae* transgene. Feeding wildtype worms *C.elegans hrm-1* or *cyk-4* RNAi consistently resulted in progeny with near 100% lethality (5/5 experiments), while worms carrying the *C.briggsae* transgenes were 80-95% resistant, indicating that the *C.briggsae* genes are functional and capable of rescuing maternal and zygotic defects. These data are summarized in Figure 19.

We verified the ability of the *C.briggsae* transgene to rescue *C.elegans cyk-4* loss of function by crossing the *cyk-4*$^{C.br}$::mCherry line into a functionally null *cyk-4* deletion PCR, and homozygous deletion mutants carrying the transgenic copy were fully viable,

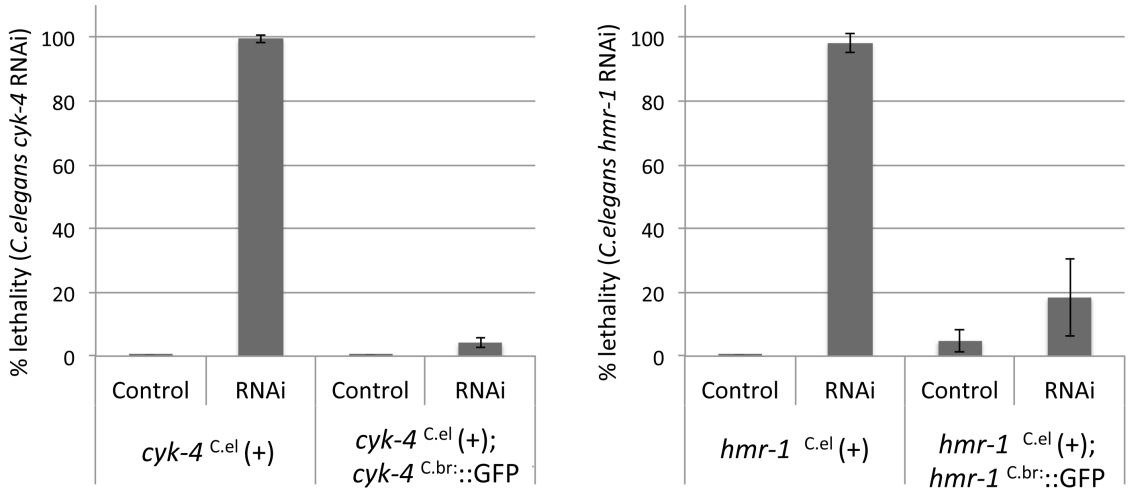


Figure 19. *C.elegans* RNAi targeting *cyk-4* and *hmr-1* induces lethality. Viability is restored if a *C.briggsae* transgene is present in single copy (*cyk-4*), or on an array (*hmr-1*).

mutant (*ok1034*). Genotypes were confirmed using poison primer PCR while homozygous deletion mutants without the transgene were never obtained, suggesting lethality. As expected, western blot analysis probing with a CYK-4 antibody shows that the endogenous protein is absent in *cyk-4*$^{C.br}$::mCherry; *cyk-4*$^{C.el}$ *(ok1034)* homozygous mutants, instead showing an upshifted band corresponding to the tagged transgene, as shown in Figure 20.

Immunofluorescence analysis demonstrated that the pattern of transgenic localization in *cyk-4*$^{C.br}$::mCherry; *cyk-4*$^{C.el}$*(ok1034)* homozygous mutants mimics the endogenous pattern, with protein observed at the spindle midzone and division remnants (20/20 embryos). An example of this stain is shown in Figure 21. Levels of transgenic protein appear similar when comparing endogenous CYK-4 stains in wildtype worms to mCherry stains in transgenic embryos, suggesting that expression levels are similar. However, in both western blots and immunofluorescence stains, a CYK-4 antibody

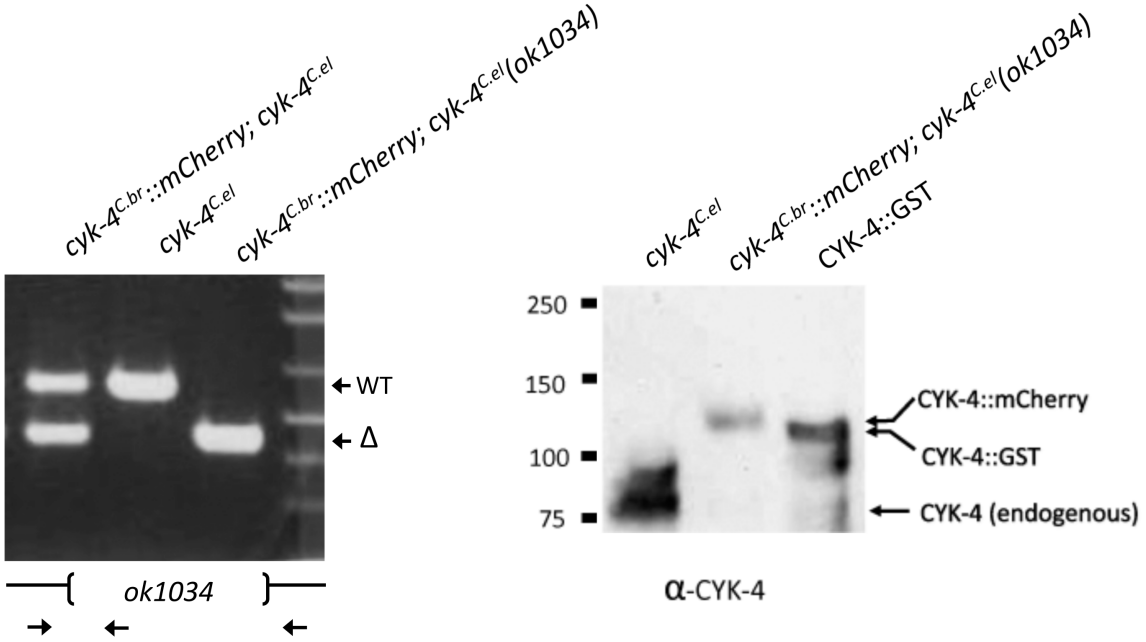


Figure 20. Genotyping and western blot of various *cyk-4* transgenic lines. Left: Poison primer genotyping (schematized below the gel) demonstrates the ability of *C.briggase* transgene in a *C.elegans cyk-4* null background. Right: Western blot using a CYK-4 antibody designed against the *C.elegans* protein. The rescueing *C.briggsae* transgene in *C.elegans cyk-4(ok1034)* background is upshifted ~50 kDa, reflecting the added weight of the mCherry fluorphore. Endogenous CYK-4 is absent in this strain. The faintness of the *C.briggsae* transgenic band may reflect reduced affinity of the *C.elegans* antibody for the transgenic *C.briggsae* protein.

generated against a *C.elegans* peptide shows a somewhat reduced affinity for the *C.briggsae* CYK-4 protein (data not shown).

To set up a system of controllable gene expression, we designed an RNAi construct to specifically target the *C.briggsae cyk-4* transgene as well as the mCherry flurophore. As previous studies demonstrated that duplexes as short as 26 nucleotides can trigger an RNAi response (Parrish et al., 2000), we looked for regions of dissimilarity and limited long stretches of base pair identity. Species specific RNAi was tested by comparing viability of C.briggsae transgenic lines (*cyk-4* $^{C.br}$::mCherry; *cyk-4*$^{C.el}$ (+) and

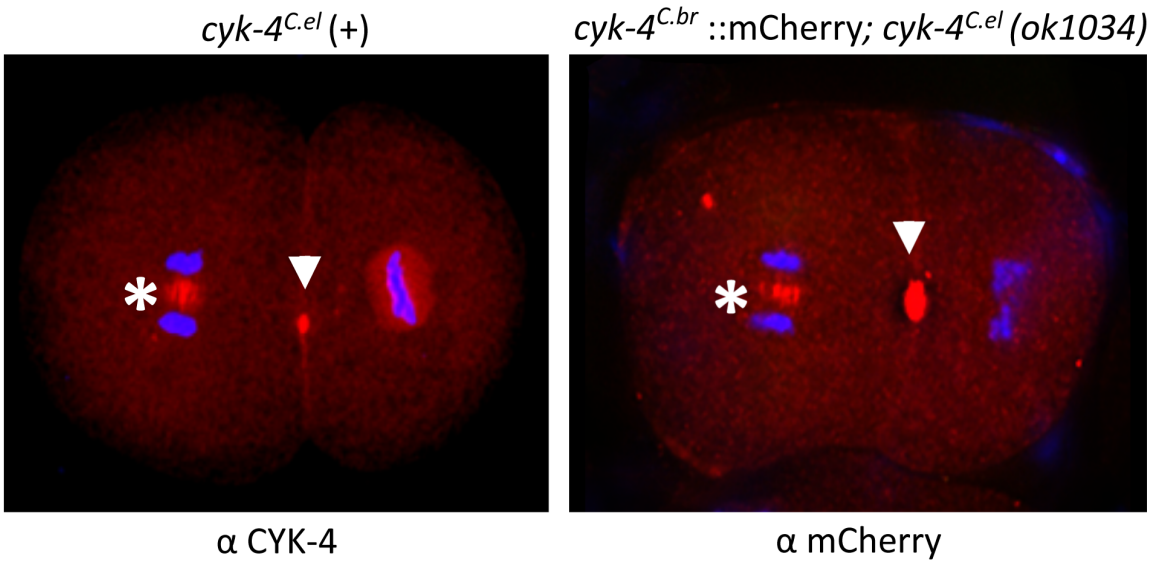


Figure 21. Immunofluorescence assays demonstrate that endogenous CYK-4 (detected with α CYK-4 antibody) and transgenic CYK-4 (detected with α mCherry antibody) localize similarly in the early embryo. Asterisks mark the spindle midzone, and arrowheads mark the division remnant. The wildtype embryo is slightly older, and the posterior cell is in metaphase, and in this cell, CYK-4 shows a slightly different staining pattern, which was also observed in transgenic embryos (data not shown).

cyk-4$^{C.br}$::mCherry; *cyk-4* $^{C.el}$ *(ok1034)*) and wildtype worms (no transgene, *cyk-4*$^{C.el}$ (+)) when grown on *C.briggsae* specific feeding RNAi relative to viability on *C.elegans* specific RNAi and control RNAi. mCherry RNAi targeting construct was also tested for efficacy of RNAi mediated knockdown. Species specific RNAi was scored by quantitation of F_1 lethality, and mRNA levels by quantified by qPCR. *C.elegans* specific RNAi was found to specifically target endogenous *C.elegans cyk-4*, as both strains carrying the *C.briggsae* transgene were fully viable (Figure 22, right columns). This finding suggests that this construct is specific, and does not cross react with *C.briggsae cyk-4* constructs. In contrast, treatment with *C.briggsae* specific or mCherry RNAi had minimal affects on wildtype worms (*cyk-4*$^{C.el}$ (+). while progeny of the transgene rescue

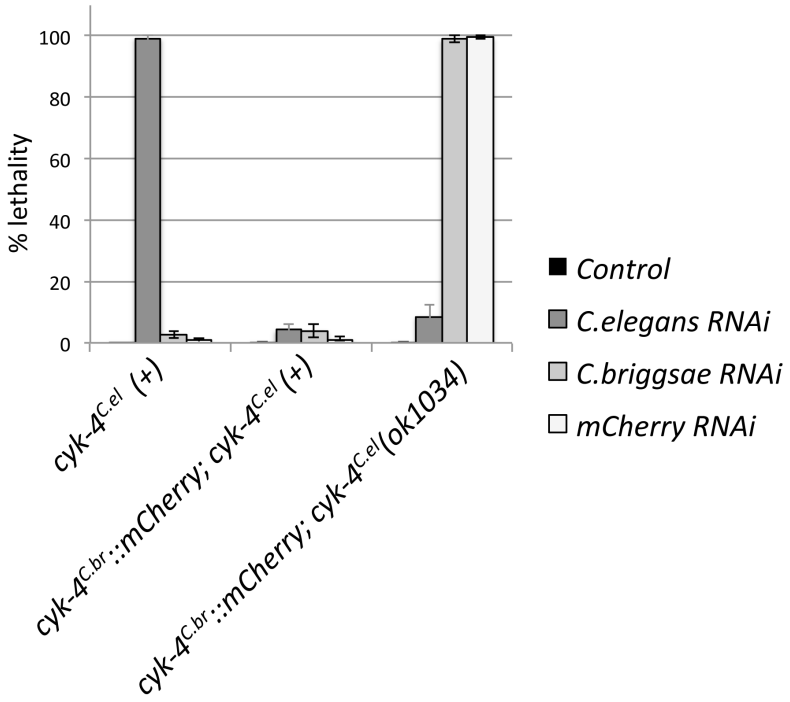


Figure 22. Species-specific RNAi constructs induce lethality in specific lines. *C.elegans*-specific *cyk-4* RNAi induces lethality in the *cyk-4*$^{C.el}$ (+), while this strain is fully viable when raised on *C.briggsae* specific RNAi. Conversely, *C.briggsae cyk-4*-specific and mCherry-specific RNAi induce 100% lethality in the *cyk-4*$^{C.br}$::mCherry; *cyk-4*$^{C.el}$ *(ok1034)* line. *cyk-4*$^{C.br}$::mCherry; *cyk-4*$^{C.el}$ (+) worms are viable on all RNAi's, presumably due to rescue by the un-targeted gene copy.

line (*cyk-4*$^{C.br}$::mCherry; *cyk-4*$^{C.el}$ *(ok1034)*) were 100% inviable in both treatments. Neither species specific or mCherry RNAi showed significant lethality in worms with both the endogenous copy and transgenic copy, suggesting that there is minimal cross reactivity between these constructs.

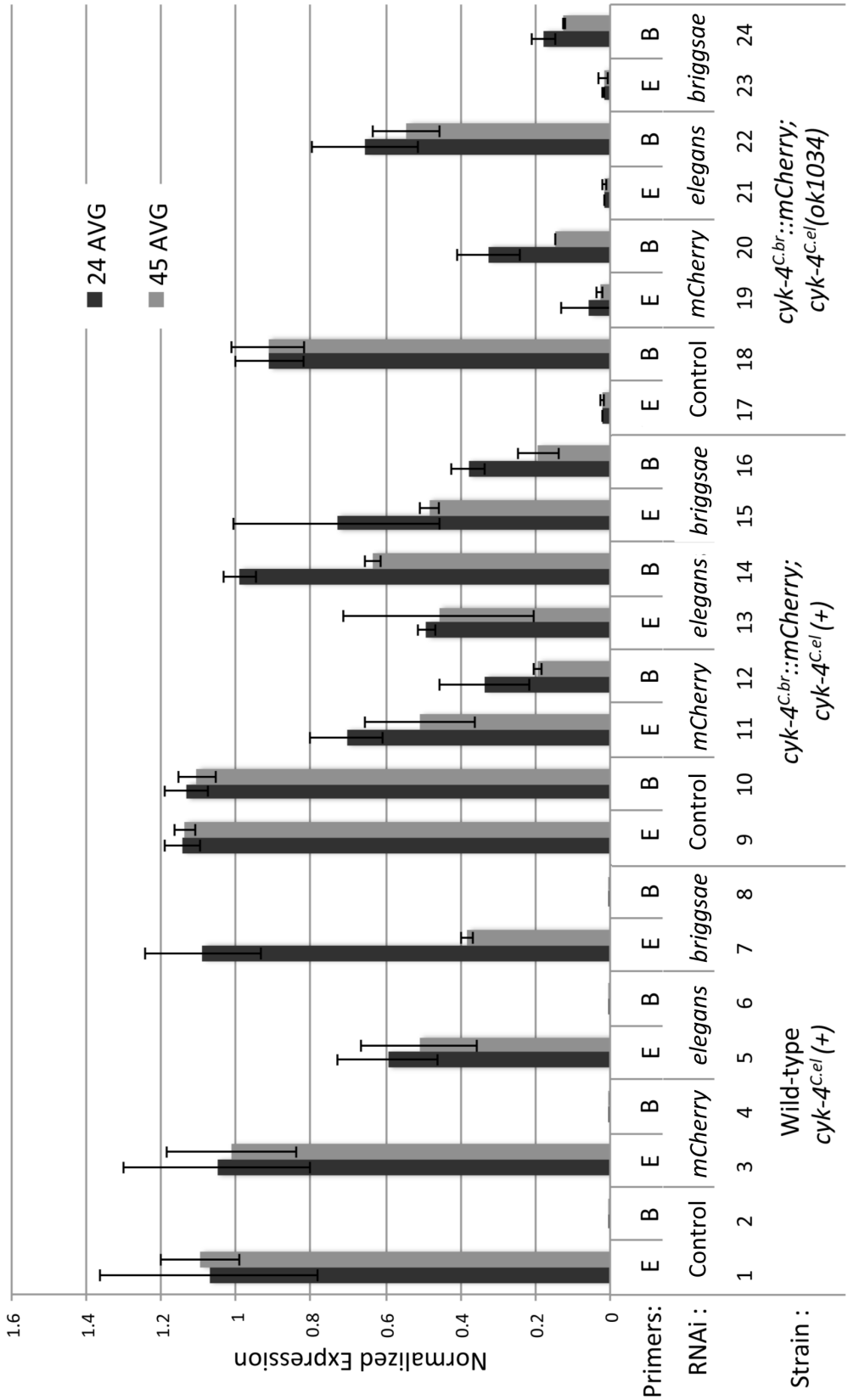
To quantify changes in mRNA levels following RNAi treatment, embryos were collected by bleaching adult mothers after 24 and 45 hours on RNAi. RNA was extracted from samples and 1 ug of RNA was used to prepare cDNA, a proportion of which (0.1 ul)

was used per qPCR reaction. Primers were designed against low conservation areas to specifically amplify either the *C.elegans* or *C.briggsae* gene copies; these primers behaved as anticipated using genomic extracts (data not shown). As shown in Figure 23, Columns 1-2, 17-18 primers are specific, and surprisingly, the transgene is expressed at a very similar level relative to the endogenous gene. After 24 hours on RNAi, treatment with *C.elegans* RNAi results in a significant decrease in the level of *C.elegans* specific transcript in wildtype (Figure 6, Column 5, pval = <0.005) and *cyk-4*$^{C.br}$::mCherry; *cyk-4*$^{C.el}$ (+), which carries both the transgenic and endogenous copy (column 13, p val = <0.005). When targeted with *C.elegans* RNAi, a significant decrease in *C.briggsae* transcript is observed in *cyk-4*$^{C.br}$::mCherry; *cyk-4*$^{C.el}$ *(ok1034)*, in which CYK-4 is expressed from the rescuing *C.briggsae* transgene (Column 22). However, these worms are viable, suggesting that the remaining level of transgene is sufficient. Similarly, treatment with *C.briggsae* RNAi reduces levels of *C.elegans* specific transcript after 45 hours (Column 7), but viable progeny are still produced. In contrast, *C.briggsae* specific and mCherry targeting RNAi constructs result in a dramatic decrease in expression of the transgene, and also result in loss of viability (Columns 24 and 20, also previous figure). Changes in transcript levels reflect a loss of protein in western blots (data not shown).

Conclusion

This work demonstrates the feasibility of a gene expression system where fluorescent labeled transgenes from the closely related nematode *C.briggsae* are introduced into the *C.elegans* genome, allowing for controlled gene expression by species specific RNAi. Previous studies suggested, but did not examine, the extent to which

Figure 23. Quantitation of mRNA levels following species-specific RNAi. *C.elegans* (E) and *C.briggsae* (B) specific primers were used to assess expression levels following control, mCherry, *C.elegans*-specific, and *C.briggsae*-specific RNAi treatments in F1 embryos at 24 and 48 hours after Po generation reached adulthood. Treatments are numbered for ease of reference within the text. Graph found on following page.


24 AVG
45 AVG
Normalized Expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Primers: E B E B E B E B E B E B E B E B E B E B E B E B
RNAi : Control mCherry elegans briggsae Control mCherry elegans briggsae Control mCherry elegans briggsae
Strain : Wild-type cyk-4C.el (+) cyk-4C.br::mCherry; cyk-4C.el (+) cyk-4C.br::mCherry; cyk-4C.el(ok1034)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

species specific RNAi efficiently depletes the endogenous or transgenic gene copy (Sarov et al., 2006). Here we demonstrate that viability is fully rescued by orthologous transgenes. mRNA levels are measured by qPCR following species specific RNAi and demonstrate the ability to targeting a specific gene copy, with minimal affects on expression of the closely related gene. This easy and effective method of controlling gene expression has many potential applications.

APPENDIX B

MULTIPLE PATHWAYS REGULATE ANTERIOR/ POSTERIOR AXIS FORMATION AT THE ONE CELL STAGE

PAR proteins and actomyosin dynamics

Polarity is a fundamental cellular trait required for diverse processes including asymmetric cell division, cell fate determination, directed cell migration, organization of epithelia, and dendrite axon specification. The *C. elegans* one cell embryo provides a well characterized system with which to study cell polarity. A group of highly conserved regulators known as PAR proteins (PARtioning defective) regulate polarization of multiple cell types in *C.elegans* and other organisms. In the *C.elegans* zygote, the anterior/posterior (A/P) axis is specified at the one cell stage in response to fertilization. Embryos become polarized along the long axis of the cell in response to sperm donated cue(s), including the centrosome. Polarization is characterized by the formation of separate PAR protein domains at the anterior and posterior poles. Proper localization of PAR proteins into disparate domains is essential for further cellular asymmetry, including the mediation of cell fate determinant localization and positioning of the mitotic spindle. Actin dynamics, specifically the asymmetric contraction of the cortical actomyosin network, is critical to establish asymmetric PAR protein localization. The molecular mechanisms by which the sperm cues

regulate actomyosin dynamics and the establishment of anterior/posterior domains remain to be fully elucidated.

PAR (PARtioning defective) proteins are key regulators of cell polarity in multiple cell types. The core PAR network includes the PDZ domain containing scaffolding proteins PAR-3 and PAR-6, the serine/threonine kinases PAR-1, PAR-4 and aPKC, the 14-3-3 protein PAR-5, and the zinc/RING finger domain containing PAR-2 (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo & Kemphues, 1995; Hung & Kemphues, 1999; Levitan et al., 1994; Morton et al., 2002; Morton et al., 1992; Tabuse et al., 1998; Watts et al., 2000; Watts et al., 1996; Cowan & Hyman, 2004; Schneider & Bowerman, 2003). With the exception of PAR-2, these core regulators are extremely well conserved throughout metazoa (Cowan & Hyman, 2004; Schneider & Bowerman, 2003).

In unfertilized embryos, PAR proteins are evenly distributed throughout the cell cortex (Rose & Kemphues, 1998; Cowan & Hyman, 2004; Cuenca et al., 2003). In response to fertilization, and following the completion of maternal meiosis, embryos become polarized by the formation of distinct domains at the cell cortex. PAR-3, PAR-6 and aPKC localize to the anterior domain (Rose & Kemphues, 1998; Cowan & Hyman, 2004). PAR-1 and PAR-2 occupy the posterior cortex (Rose & Kemphues, 1998). PAR-4 and PAR-5 remain localized throughout the cortex and cytoplasm (Rose & Kemphues, 1998; Cowan & Hyman, 2004). Proper localization of PAR proteins is essential for subsequent cellular asymmetries.

Prior to fertilization, oocytes are arrested in meiotic prophase I, and display no asymmetry with respect to PAR proteins, cell fate determinants, or cortical actomyosin (Guo & Kemphues, 1996a; Rose & Kemphues, 1998; Goldstein & Hird, 1996; Cowan & Hyman, 2004). Fertilization triggers the completion of maternal meiosis and dramatic

rearrangement of the cortex and cytoplasm (Strome & Wood, 1983; Strome & Hill, 1988; Hird & White, 1993; Goldstein & Hird, 1996). Just prior to the completion of meiosis II, the meshwork of actomyosin associated with the cell cortex (observed with non-muscle myosin II heavy chain fused to GFP (NMY-2::GFP, Munroe et al., 2003) becomes highly dynamic, forming contractile foci that rapidly assemble, move short distances and dissociate or merge with other foci (Munro et al., 2003). Formation of these contractile foci coincides with transient membrane invaginations ("ruffling") in the cell cortex. These local contractions suggest that the cortical actomyosin network stably exists under uniform dynamic tension (Munro et al., 2004).

Polarization initiates following the completion of maternal meiosis II. The first cellular asymmetries are observed when the paternal pronuclear associated centrosomes physically contact the posterior cell cortex. At this point, NMY-2::GFP foci cease to form in the cortical region nearest the pronucleus/centrosome (Munro et al., 2004). As a whole, the actomyosin network and existing foci move collectively toward the anterior (Munro et al., 2004). This data is supported by previous reports of an "anterior cap" of endogenous concentrated actomyosin (Strome, 1988; Kirby et al., 1990). The relocalization the cortical actomyosin is suggestive of localized relaxation in the posterior, leading to contraction of the actomyosin network toward to anterior. A smooth cortical domain forms in the posterior, which ultimately spreads to occupy approximately 50% egg length (Munro et al., 2004). To summarize the current model, initiation of polarity induces a local change in actomyosin dynamics in the posterior, which results in a gradient of actomyosin contractility and the wave of actomyosin that moves toward to anterior.

The anterior PAR proteins relocalize to the anterior during the period of cortical flow that establishes the anterior actomyosin cap (Munro et al., 2004; Etemad-Moghadam et al., 1995). The cortical localization and asymmetrical domain formation of anterior PAR proteins requires actomyosin dynamics (Munro et al., 2004; Etemad-Moghadam et al., 1995; Cuenca et al., 2003). Movement of anterior PARs tracks very tightly with the anterior relocalization of NMY-2 (Munro et al., 2004). In wildtype embryos, physical contact between the centrosome and posterior cortex is followed by cessation of contractile actomyosin foci formation. Coincident with clearing of contractile machinery, the PAR-3/PAR-6/aPKC complex begins to delocalize from the posterior cortex (O'Connell et al., 2000; Cuenca, et al., 2004; Cowan & Hyman, 2004; Munro et al., 2004; Wallenfang & Seydoux, 2000). PAR-3 and PAR-6 translocate with contractile foci as the actomyosin network sweeps toward the anterior (Etemad-Moghadam et al., 1995; Cheeks et al., 2004; Munro et al., 2004; Cuenca et al., 2003; Jenkins et al., 2006). The anterior PAR domain is fully established approximately ten minutes following the onset of posterior smoothing (Cowan & Hyman, 2006; Cuenca et al., 2004; Munro et al 2004).

During maternal meiosis, and prior to the onset of polarity, PAR-2 localizes transiently to the cortical region overlying the meiotic spindle (generally in the presumptive anterior)(Cuenca et al., 2003; Boyd et al., 1996). Lagging slightly behind the relocalization of PAR-3/PAR-6 to the anterior, but within two minutes of the paternal pronuclear associated centrosomes contacting the posterior cortex, PAR-2 begins to accumulate in the smooth posterior domain (Guo & Kemphues, 1995; Boyd et al., 1996; Cuenca et al., 2003; Cowan & Hyman, 2004). The smooth, PAR-2 associated posterior domains expands during the ten minute period which precedes pseudocleavage (Guo &

Kemphues, 1995; Boyd et al., 1996; Cuenca et al., 2003; Cowan & Hyman, 2004). PAR-1 colocalizes with PAR-2 at the posterior cortex, a localization pattern that is PAR-2-dependent (Boyd et al., 1996).

Embryos treated with Latrunculin A or Cytochalasin D show severe polarity defects with respect to both contractile asymmetry and cortical PAR domain formation (Severson & Bowerman, 2003; Hill & Strome, 1988, Hill & Strome, 1990; Severson et al., 2002). Loss of polarity is also associated with the RNAi mediated depletion of actomyosin cytoskeletal components, including NMY-2 (non-muscle myosin II heavy chain), the myosin regulatory light chain MLC-4, and profilin (PFN-1), an actin binding protein required for the cortical accumulation of microfilaments (Severson et al., 2002). In *nmy-2*, *mlc-4* or *pfn-1* RNAi treated embryos, PAR-3 is mislocalized throughout the cortex and PAR-2 cortical localization lost entirely (Guo & Kemphues, 1996; Shelton et al., 1999; Severson & Bowerman, 2003; Cuenca et al., 2003), indicating that a functional actomyosin network is required for the anterior pullback of PAR-3, and may also be responsible for the cortical localization of PAR-2.

The Rho family of guanosine triphosphatases (GTPases) includes RhoA, Rac, and Ccd42. Rho family members act as molecular switches, cycling between the GTP-bound (active) and GDP- bound (inactive) states to control a variety of biological processes involving actin dynamics. Rho regulates the assembly of contractile actomyosin filaments that provide the mechanical force required to drive cell migration, cortical ruffling, and cytokinesis (Etienne-Manneville & Hall, 2002). Rac and Cdc42 regulate the polymerization of actin to form lamellipodia and filopodia, respectively (Etienne-Manneville & Hall, 2002).

RhoA-GTPase is essential for early polarity establishment in *C. elegans*. In *rho-1/RhoA* RNAi depleted embryos, the actomyosin network fails to form normally, contractile polarity is abolished, anterior PAR proteins are mislocalized throughtout the cell cortex (Schonegg & Hyman, 2006; Motegi & Sugimoto, 2006; Jenkins et al., 2006). The downstream effector MLC-4 is also required for A/P polarization in the one cell embryo, as genetic knockdown induces mislocalization of PAR-3 throughout the cortex (Shelton et al., 1999). MLC-4 is also required for the polarized cytoplasmic flow directed at the paternal pronucleus/centrosome prior to apposition at the posterior cortex (Shelton et al., 1999). Phospho-MLC, indicative of activated myosin light chain, colocalizes with the dynamic NMY-2 foci prior to fertilization (Jenkins et al., 2006). In the absence of *rho-1*, phospho-MLC is not detected at the cell cortex, indicating that Rho-1 promotes assembly or activity of the actomyosin contractile apparatus by inducing the phosphorylation of MLC-4.

The RhoGEF ECT-2 (Pebble) localizes throughout the cell cortex prior the initation of polarity, and is required for A/P axis specification. Loss of function phenotypes associated with *ect-2* RNAi are similar to the loss of Rho-1, including the mislocalization of anterior PARs and a failure to form the dynamic contractile network (Motegi & Sugimoto, 2006; Jenkins et al., 2006). These data indicate that the activation of Rho-1 via ECT-2 (GEF) is required to set up the cortical contractile network required for anterior PAR localization. ECT-2 and RHO-1 function to establish stable contractile meshwork such that tension is uniform throughout the cell cortex.

Following establishment of the disparate A/P PAR domains, mutual inhibition between the anterior and posterior associated proteins stably maintains the cellular

asymmetry. This 'maintenance phase' of polarity begins just after the meeting of the maternal and paternal pronuclei. The anterior complex appears to antagonize the posterior domain, as loss of *par-6*, *par-3*, or *pkc-3* leads to mislocalization of PAR-1 and PAR-2 throughout the cell cortex (Etemad-Moghadam et al., 1995; Watts et al., 1996; Tabuse et al., 1998; Hung & Kemphues, 1999; Cuenca et al., 2003). Establishment of the anterior domain does not initially require PAR-2, as the anterior pullback of PAR-6::GFP occurs even when PAR-2 has been depleted by RNAi. PAR-2 is required for domain maintenance however, as the anterior domain collapses back toward the posterior in the absence of PAR-2 (Cuenca et al., 2003). PAR-2 localization at the cell cortex is inhibited by an aPKC kinase dependent mechanism (Boyd et al., 1996; Cuenca et al., 2003; Kemphues, 2000).

PAR protein localization also feeds back to regulate actomyosin dymanics during the maintenance phase of polarization. PARs -3, -6, and -5 are required for cortical flow, and also contribute to the localization of the anterior complex (Etemad-Moghadam et al., 1995; Watts et al., 1996; Tabuse et al., 1998; Hung & Kemphues, 1999; Cuenca et al., 2003). PAR-2 inhibits the cortical localization of NMY-2 following the formation of the posterior domain (Tabuse et al., 1998; Hung and Kemphues; 1999; Etemad-Moghadam et al., 1995; Boyd et al., 1996; Cuenca et al., 2003). These data suggest that maintenance of A/P domains involves mutual inhibition between the anterior and posterior PARs.

The centrosome as a polarity cue

Centrosomes are eukaryotic organelles that function as the major microtubule organizing center (MTOC) of the cell, responsible for the organization of bipolar spindles

during mitosis. A centriole pair forms the core of the centrosome. Centrioles are cylindrical, microtubule based structures with 9 fold radial symmetry (Palazzo, 2002). A mature centrosome is comprised of a centriole pair, the matrix, in which the centriole pair is orthogonally embedded and flexibly held together, and the electron dense pericentriolar material (PCM), which surrounds the centriole pair. The PCM is made up of numerous protein components, including γ-tubulin, which nucleates and anchors microtubules (Hamill et al., 2002; Palazzo, 2002).

In addition to the paternal complement of DNA, sperm donate a centriole pair to the oocyte, which lacks centrosomes. Following fertilization, the sperm derived centrioles separate and each duplicates to form an adjacent daughter centriole (Kirkham et al., 2003). Each new centriole pair recruits PCM components from the maternal cytoplasm, such that a mature centrosome exists at each spindle pole by the onset of first mitotic metaphase (Kirkham et al., 2003).

The sperm donated centrosome is critical to the induction of the A/P axis (Goldstein & Hird, 1996; Sadler & Shakes, 2000). The centrosome, donated as a pair of centrioles, is required for the formation of both the anterior and posterior domains, while the Rho-GTPase activating protein (GAP) CYK-4, also donated by the sperm, is required for the formation of the anterior cortical domain (Cowan & Hyman, 2004; O'Connell, et al., 2000; Jenkins et al., 2006). These cues likely act upstream of changes in actomyosin dynamics, as asymmetric actomyosin contraction is blocked in cyk-4 loss of function and ablation or blocking of centrosome maturation. Specification of body axis in response to sperm donated cues is not unique to *C. elegans*. Sperm also initiate polarization in *Xenopus* (Gerhart et al., 1981; Scharf & Gerhart, 1980) and mouse (Piotrowska &

Zernicka-Goetz, 2001), although the latter is more controversial. It is unknown whether sperm induced polarization in these species occurs by reorganization of actomyosin.

Several centrosome proteins are essential for the assembly and function of a mature centrosome, including the coiledcoil proteins SAS-4, SAS-5, SAS-6, and the atypical kinase ZYG-1 (O'Connell et al., 2001; Kirkham et al., 2003; Delattre et al., 2004; Leidel & Gonczy, 2003; Leidel et al., 2005). SPD-2, localized to centrioles as well as the PCM, as well as the PCM components SPD-5, the Aurora kinase AIR-1, the microtubule nucleator γ-tubulin, and the ring structure complex ZYG-9/TAC-1 are also required (Pelletier et al., 2004; Bellanger et al., 2003; Hannak et al., 2002; O'Connell, 2000; Hamill et al., 2002; Kemp et al., 2004; Matthews et al., 1998).

While many of the essential centriole/centrosome components are paternal, many additional centrosome and PCM components are maternally acquired, and begin to be recruited to the centrosome prior to the completion of maternal meiosis (Dammerman et al., 2004; Pelletier et al., 2006). Several maternal components are loaded prior to the onset of polarity (Hannak et al., 2002; Schumacher et al., 1998; Wallenfang & Seydoux, 2000; Mango Lab)

Several lines of evidence indicate that the sperm donated centrosome provides at least one polarizing cue. First, the pole proximal to the site of sperm entry, occupied by the paternal pronuclear associated centrosomes, always becomes the posterior (Goldstein & Hird, 1996). Second, anucleate sperm, which retain the centriole pair, are capable of polarizing the one cell zygote (Sadler & Shakes, 2000). Third, asymmetries in both acytomyosin contractility and PAR protein localization are initiated when the centrosomes come into physical contact with the posterior cortex (O'Connell, et al.,

2000; Cheeks et al., 2004; Cuenca et al., 2003; Munro et al., 2004; Cowan and Hyman, 2004). Polarity is abolished when physical contact is inhibited by defects in fatty acid synthesis or APC impairment (Rappleye et al., 2002; Rappleye et al., 2003). Fourth, inhibition of centrosome maturation (ie, inhibition of centriole assembly or acquisition of essential PCM components) via RNAi-mediated knockdown of essential components leads to polarity defects with respect to both cortical PAR domain formation and actomyosin contractile asymmetry. Similarly, loss of *spd-2* or *air-1* leads to mislocalization of PAR-3 throughout the cell cortex (O'Connell et al., 2000; Wallenfang & Seydoux, 2002). The absence of *spd-2* or *spd-5* also causes defects in the posterior clearing of NMY-2 (Munro et al., 2004). Fifth, ablation of the centrosome prior to onset of posterior smoothing inhibits the formation of the PAR-2 domain, confirming that the centrosome or an associated component is required for the initiation of polarity. Ablation of the centrosome during the formation of PAR-2 domain resulted in a reduced posterior domain, while ablation of the centrosome following establishment of the PAR-2 domain does not affect domain formation or maintenance (Cowan and Hyman, 2004), suggesting that the polarizing signal from the centrosome is transient, but essential for the initiation of A/P polarity.

Given that the establishment of A/P polarity is an actomyosin dependent process, how does the centrosome exert its effects on actin to control cell polarity? Although hotly debated, current evidence argues that the role of the centrosome in polarity is independent of its role in microtubule organization. Nocodozol induced depolymerization of microtubules does not affect asymmetric A/P cortical domain establishment (Strome & Wood, 1983; Hyman & White, 1987; Strome & White, 1993;

Hird & White, 1993). Pharmacological depolymerization data is confirmed by genetic knockdown of tubulin components. RNAi targeting α-tub, β-tub, γ-tub, or γ-tub ring structure components abrogates the nucleation of microtubule asters normally observed in the one-cell zygote (Sonneville & Gonczy, 2004; Cowan & Hyman, 2004). However, polarization of the A/P axis remains intact, with normal formation of the anterior (PAR-3, PAR-6) and posterior (PAR-1, PAR-2) domains (Sonneville & Gonczy, 2004; Cowan & Hyman, 2004). In addition, the centrosome cue appears to function upstream of actomyosin relaxation in the posterior, as depletion of centrosome components cause defects in the relocalization of NMY-2::GFP (Munro et al., 2004).

However, the idea that the centrosome cue is microtubule independent remains highly controversial, as other groups contend that microtubules are necessary and sufficient to initiate polarity. This argument is based on the finding that inhibition of centrosome maturation using RNAi or *mat* mutants leads to a reversal of the A/P axis with respect to PAR domains and some cell fate determinants (PIE-1 but not p-granules) (Wallenfang and Seydoux, 2000; O'Connell et al., 2000). The authors theorize that the accumulation of "posterior" factors near the arrested/degrading meiotic spindle indicate that the polarizing signal is provided by microtubule plus end contact with the cell cortex, a signal generally provided more robustly by the sperm associated centrosomes. This "reversed" polarity is sensitive to nocodozole, suggesting that this break in symmetry may also be microtubule dependent (Wallenfang & Seydoux, 2002; Tsai & Ahringer, 2007). More recent studies demonstrate in live embryos demonstrate a strong correlation between the presence of a parental nucleus associated microtubule aster and the onset of axis formation (Tsai & Ahringer, 2007). In this study, knockdown of tubulin resulting in

delayed in aster formation also resulted in delayed polarity onset (Tsai & Ahringer, 2007). However, it has also been argued that complete abrogation of microtubules in the one cell embryo is technically challenging, and persistent astral microtubules may influence polarization. The opposing view asserts that these attenuated microtubules would not be capable of transmitting polarization cues.

The centrosome plays a clear role in the initiation of A/P polarity. However, the specific centrosomal component(s) involved, as well as the mechanism of polarity initiation remains ambiguous Inhibiting centrosome maturation prevents not only nucleation and organization of astral microtubules, but also inhibits the acquisition of numerous PCM components. Thus, the key questions remains; what component(s) of the centrosome are necessary to initiate polarity?

One intriguing candidate is AIR-1. Aurora-A kinase (AIR-1 in *C. elegans*), localizes to centrosomes and astral microtubules, and is essential for assembly of the bipolar mitotic spindle, as well as centrosome maturation and maintenance of separation (Hannak et al., 2001; Schumacher et al., 1998; Ferrari, 2006). Genetic loss or mutation of AIR-1 leads to embryonic lethality due to severe aneuploidy (Schumacher et al., 1998). Aneuploid cells display disorganized mitotic spindles, abnormal centrosomes, and string-like or hypercondensed chromatin (Schumacher et al., 1998). AIR-1 is required for the recruitment of multiple PCM factors, including the kinase zyg-9, CeGrip, TACC, centrosomin (Cnn), and the microtubule nucleator y-tub, which is reduced ~60% in *air-1 (RNAi)* embryos (Hannak et al., 2001; Giet et al., 2002; Terada et al., 2003). Recruitment of these molecules appears to be microtubule independent (Hannak et al.,

2001). Nuclear envelope breakdown (NEB) is delayed by one to two minutes (relative to other mitotic events) in *air-1 RNAi* embryos (Hannak et al., 2001).

Aurora-A also functions in the generating cellular asymmetry. *C. elegans* embryos depleted of *air-1* have defects in the asymmetric distribution of germ line determinants, including PIE-1 and p-granules (Schumacher et al., 1998). In addition, the anterior PAR-3 is mislocalized throughout the cell cortex (Wallenfang & Seydoux, 2003). Another example of Aurora based regulation of cell fate determinant asymmetry comes from experiments using Drosophila sensory organ precursor (SOP) cells. In these cells, the cell fate determinant Numb is asymmetrically localized to the anterior cortex and segregated into the neuronal precursor cell. Aurora-A is required for this asymmetric segregation, and appears to be independent of the role of Aurora in centrosome function (Berdnik & Knoblich, 2002). Aurora-A members are emerging as key regulators of cellular asymmetry by controlling actomyosin dependent segregation of cell fate determinants (Hannak et al., 2001; Berdnik & Knoblich, 2002).

CYK-4 as a sperm donated polarity cue

In *C. elegans* zygote, the RhoGAP CYK-4 also acts as a sperm donated polarity cue, likely acting on RhoA and influencing the actomyosin network and anterior PAR domain formation (Jenkins et al., 2006). CYK-4 is dramatically enriched in sperm and is released into the presumptive posterior pole of the egg upon fertilization. Paternally donated CYK-4 is positionally and temporally localized to the future posterior of the embryo, where it could function as a polarity cue. In the posterior, this bolus of CYK-4 forms punctuate structures that associate with sperm donated membranous organelles, the

paternal pronucleus, and the posterior cortex during maternal meiosis and through the onset of polarity (Jenkins et al., 2006). The human ortholog of CYK-4 (male germ cell Rac GAP (mgcRac GAP)) is enriched in male germ cells, as is the Drosophila counterpart, Rotund Rac GAP, (RnRacGAP) (Toure et al., 1998; Agnel et al., 1992), suggesting that this polarizing cue may be evolutionarily conserved.

RNAi mediated knockdown of paternal CYK-4 induces mislocalization of anterior PAR proteins, indicating that the CYK-4 cue controls formation of the anterior domain (Jenkins et al., 2006). Scored at pronuclear meeting, PAR-6::GFP extends toward the anterior to occupy 87% egg length, as opposed to 47% egg length in wildtype embryos (Jenkins et al., 2006). In these embryos, endogenous PAR-3 is also mislocalized throughout the cortex (Jenkins et al., 2006). PAR-2 localization is relatively wildtype, although a reduction in the size of the posterior domain is observed (~30% compared to 405 in wildtype) (Mango lab, unpublished). The maternal component of CYK-4, required for maternal meiosis as well as the first mitotic cytokinesis, is not required for the initiation of A/P polarity (Jenkins et al., 2006).

The role of CYK-4 in the localization of PAR proteins likely occurs by regulation of the actomyosin contractile network. In the absence of paternal CYK-4, the actomyosin network remains contractile over the entire cortical surface (Jenkins et al., 2006). These data suggest that CYK-4 acts upstream of actomyosin relaxation and influences the formation of the anterior PAR domain. Our data indicate that paternal CYK-4 controls the actomyosin network by negatively regulating the small GTPase Rho-1/RhoA. The net effect of Rho-1 down regulation is local relaxation of the actomyosin network in the posterior (Jenkins et al., 2006).

Results and discussion

We hypothesized that the sperm donated bolus of CYK-4 GAP poised in the posterior induces a local relaxation, resulting in a gradient of tension and the wave of actomyosin, leading to the relocalization of cortically associated PAR proteins to form the anterior domain. We proposed a model of A/P axis specification in which the two sperm donated cues (the centrosome and CYK-4) act in a common pathway to control the formation of the anterior cortical protein domain. In this model, a component of the centrosome, specifically the Aurora A kinase AIR-1, activates CYK-4 via phosphorylation, leading to an increase in GAP activity toward the small GTPase RHO-1/RhoA, a key regulator of actin dynamics essential for A/P polarity induction in the one cell embryo.

This model was based on a paradigm from cytokinesis, in which CYK-4 was initially identified and studied (Jantsch-Plunger et al., 2000; Gonczy, 1999; Jantsch-Plunger et al., 2000; Hirose et al., 2001; Ban et al., 2004). Studies have demonstrated a phosphorylation based mechanism by which the CYK-4 ortholog mgcRacGAP to increases its latent GAP activity toward RhoA. These studies have demonstrated that serine residues of mgcRacGAP are phosphorylated by Aurora B At the central midbody, a microtubule based structure derived from the central spindle (Minoshima et al., 2003). Aurora B is an Aurora family kinase functioning during mitosis, and localizes to mitotic chromosomes and the central midbody during late stages of mitosis and cytokinesis (Kitamura et al., 2001; Minoshima et al., 2003). During telophase, direct interaction between Aurora B and mgcRacGAP results in mgcRacGAP becoming phosphorylated (Minoshima et al., 2003). *In vitro* phosphorylation assays indicate that this

phosphorylation effects results in a dramatic increase in the ability of mgcRacGAP to hydrolyze GTP from RhoA, greater than 70% compared to unphosphorylated (Minoshima et al., 2003). Phosphorylation by AuroraB does not affect the GAP activities toward Cdc-42 and Rac (Minoshima et al., 2003; Ban et al., 2002). Serine 387, located in the highly conserved GAP domain, was identified as the critical residue for this phosphoswitch, although additional residues, including S387 and S410 are also phosphorylated by Aurora B (Minoshima et al., 2003; Ban et al., 2002). These findings were confirmed *in vivo* using phosphospecific antibodies, kinase dead Aurora B, and phosphomimetic (S387D) and phosphodefective (S387A) versions of mgcRacGAP (Minoshima et al., 2003).

Based on these findings, we postulated that Aurora kinase AIR-1, localized at the centrosome, would be poised to phosphorylate the posterior localized bolus of CYK-4. This activation event would then trigger the locale decrease in actomyosin contractility by decreased activity of RhoA GTPase. This model would account for the discrepancy between *in vivo* data, implicating RhoA as the likely target of CYK-4, and *in vitro* data, reporting the negligible GAP activity of CYK-4 toward Rho-1. Both human and worm orthologs of CYK-4 show greater GAP activity toward cdc-42 and Rac than toward RhoA (Jantsch-Plunger et al., 2000; Toure et al., 1998). Others have reported that CYK-4/mgcRacGAP mediated GTP hydrolysis from RhoA is below the level of detection (Kawashima et al., 2000; Raymond et al., 2001). This model would also account for the 30 minute lag time observed between fertilization and the onset of A/P axis formation.

Aurora A (AIR-1) kinase is localized to centrosomes and is required for maintenance of centrosome separation and recruitment of y-tubulin (Hannak et al 2002).

To address whether AIR-1 is present and active at the onset of polarization, immunofluorescence assays were used to examine specific stages of A/P axis formation. Previous studies have demonstrated that loss of AIR-1 function results in polarity defects (Wallenfang and Seydoux, 2002; O'Connell et al., 2000), although whether this effect was due to failure of centrosome maturation and function was not addressed.

To determine whether AIR-1 might function as a centrosomal effector of polarity and/or CYK-4 function, antibody stains were used to detect AIR-1 localization at the centrosome during the early stages of A/P polarization. As we predicted that the role of AIR-1 would depend on kinase activity, we also tested the activation status of AIR-1 using a phosphospecific antibody (Thr288-PO_4, Cell Signaling, d13a11), and total AIR-1 was assayed using a *C.elegans* specific AIR-1 antibody generated and generously provided by the Oegema lab (UCSD). Antibody stains were performed using standard protocols, and were costained with polarity markers, including PAR-3, PAR-6::GFP and NMY-2::GFP. The stages associated with polarity initiation were defined by pronuclear size and localization, DNA condensation of the paternal pronucleus, meiotic status of the maternal DNA, and the localization of anterior polarity markers. The anterior pole was generally defined by maternal meiosis and/or the resulting polar body, and the paternal pronucleus was generally observed in the posterior. As shown in Figure 24, AIR-1 total protein is initially high in the cytoplasm, and is recruited to the centriole pair during maturation and duplication of the centrosomes, which is associated with increased size of

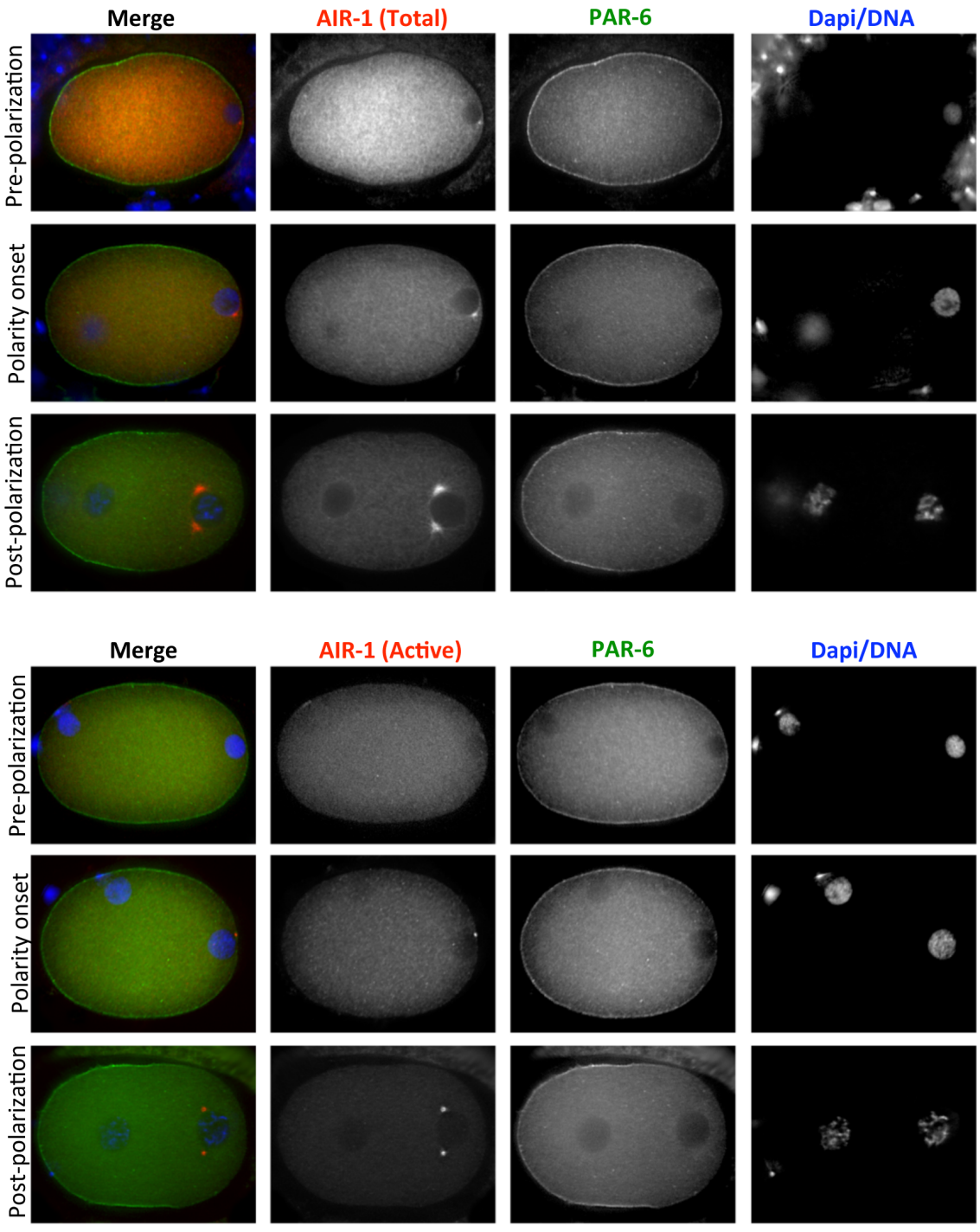


Figure 24. AIR-1 total protein and active AIR-1 antibody staining during A/P polarization.

the male pronucleus. At this stage, the paternal pronucleus was often, but not always, associated with the posterior cortex. Over time, levels of AIR-1 total protein in the cytoplasm decrease, concomitant with an increased size of centrosomes. At the onset of polarity, fairly sizable but generally separated centrosomes were juxtaposed to the cortex, and if localized to one side of the pronucleus, this side of the embryo inevitably showed stronger clearing of anterior marks. In contrast, Thr288-PO_4 AIR-1 (activated) is extremely low in the cytoplasm, and localizes (or more likely is activated) at the centrosome just prior to, or coincident with the onset of polarity initiation. As shown in Figure 25, >95% of embryos (N=43) have detectable activation of AIR-1 at the centrosome, while AIR-1 total is localized in earlier stages. While some factor(s) responsible for activation of AIR-1 have been suggested in other species (i.e., TXP, Bora), the kinase has not been identified in C.elegans, and thus was not tested for a role in polarity (Karsenti, 2005; Ozlu et al., 2005).

As shown in Figure 26, activated AIR-1 occupies a small domain in the interior of the centrosome, likely associated with the centriole pair. These data demonstrate that AIR-1 is spatially and temporally highly regulated at the centrosome, and that activation strongly correlates with the onset of polarity. Based on these findings, AIR-1 kinase is a strong candidate to activate CYK-4 GAP activity at the posterior cortex, inducing the local decrease in RhoA GTPase function and actomyosin contractility.

We next wanted to examine the polarity defects associated with loss of AIR-1 function, based on the hypothesis that if AIR-1 functions upstream of CYK-4, loss of these factors might have similar polarity phenotypes. While we were unable to replicate previous published *cyk-4* RNAi results (Jenkins et al., 2006), we did observe that *air-1*

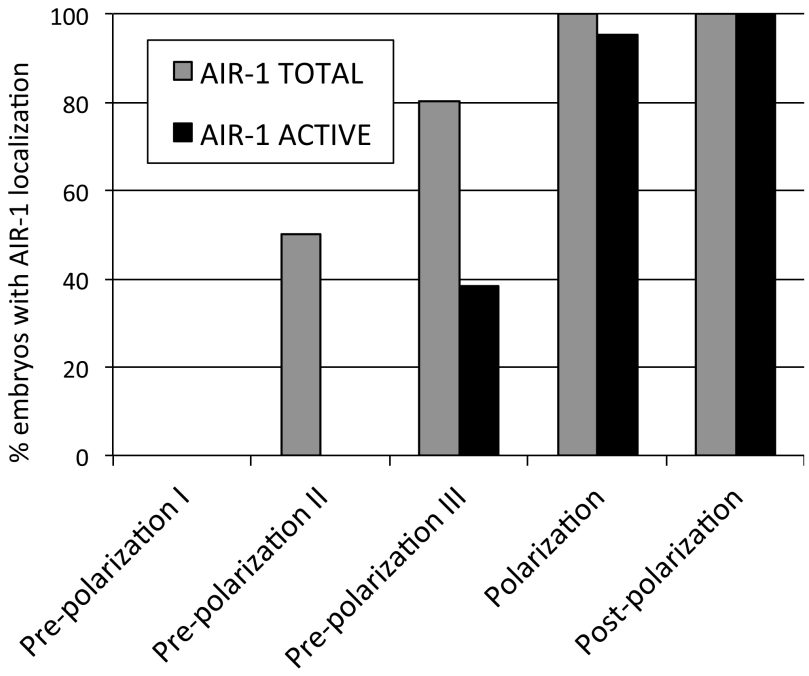


Figure 25. Quantitation of AIR-1 localization during polarity onset. In prepolarization I stage embryos, anterior polarity markers are localized throughout the cortex, the maternal pronucleus is in meiosis I, and the paternal pronucleus is highly condensed. Prepolarization II is characterized by maternal meiosis I/II, extrusion of the first polar body, and enlargement of the male pronucleus, but the absence of DNA condensation. In prepolarization III, one or both polar bodies are extruded at the anterior and the male pronucleus is slightly larger but lacking DNA condensation. Polarization is characterized by localization of the male pronucleus at the posterior cortex, roughly equivalent male and oocyte pronuclear size, condensation of the pronuclear DNA, extrusion of both polar bodies at the anterior, and the partial clearing of anterior markers from the posterior pole. Postpolarization stage embryos have more complete posterior clearing, obvious DNA condensation, and pronuclear migration toward the center of the embryo.

RNAi resulted in variable polarity phenotypes. As shown in Figure 27, antibody staining of fixed embryos demonstrates highly reproducible defects. In control RNAi treated embryos, anterior markers (PAR-6::GFP, NMY-2::GFP, PAR-3) are relocalized to the anterior domain in the period following A/P polarity onset and pronuclear migration. Following *air-1* RNAi treatment, embryos show either complete mislocalization of

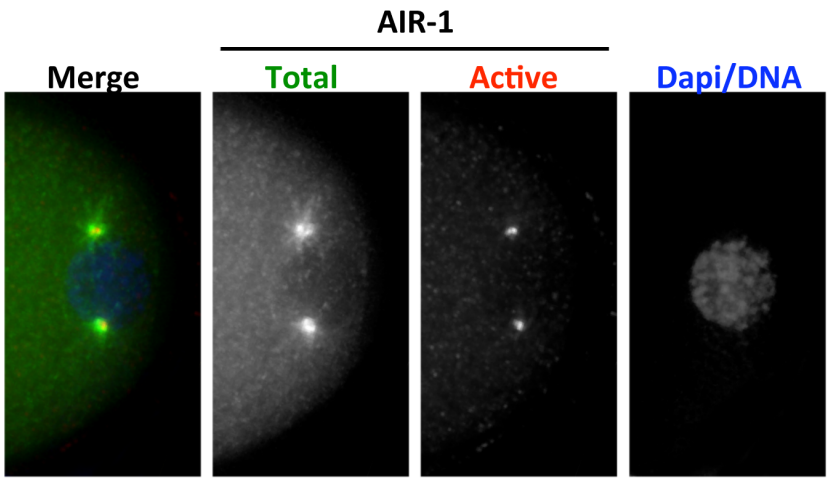


Figure 26. Activated AIR-1 is localized with a subset of AIR-1 total protein, while AIR-1 total also localizes to astral microtubules.

anterior markers (28%), reversed polarity, in which anterior markers are removed from the anterior cortex, perhaps in response to components associated with residual meiotic spindle (28%), bilateral clearing, in which small domains of anterior markers are cleared from both poles (27%), and anterior markers in the wildtype configuration but extended toward the midline and into the posterior domain.

To address whether these variable phenotypes could be attributed to weak or partial RNAi knockdown, we examined the AIR-1 localization and activity by staining AIR-1-PO_4 and microtubules. As shown in Figure 28, >95% of *air-1* RNAi treated embryos lack detectable AIR-1 activity and >90% fail to form microtubule asters during the early stages of polarization. A proportion of late stage embryos, assayed at pronuclear meeting, form microtubules asters that fail to separate and appear wiggly, disorganized, and off axis. The associated polarity phenotypes do not seem to correlate in any specific pattern with these changes. We did not address however, possible delays

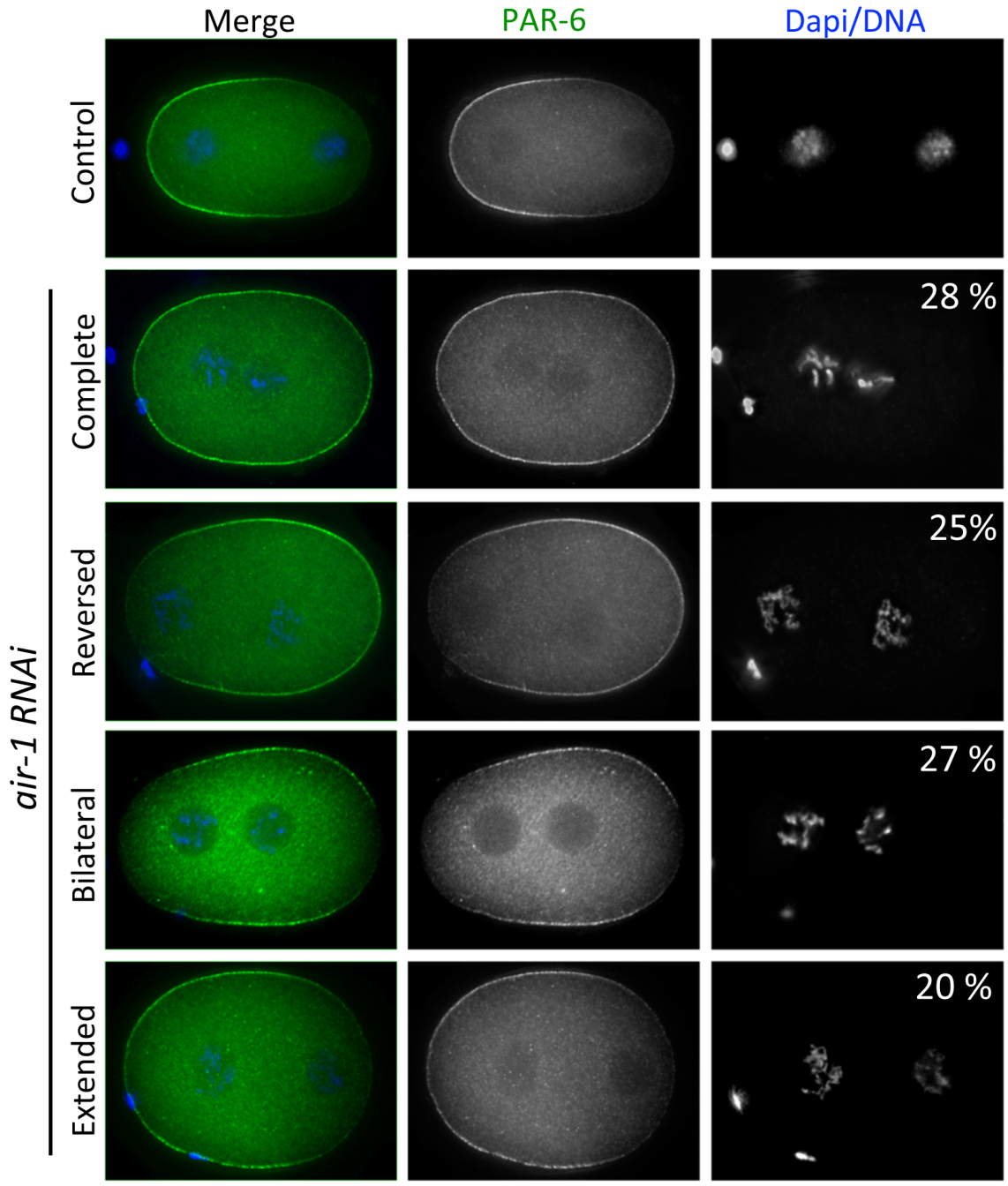


Figure 27. Loss of AIR-1 function results in a range of polarity phenotypes.

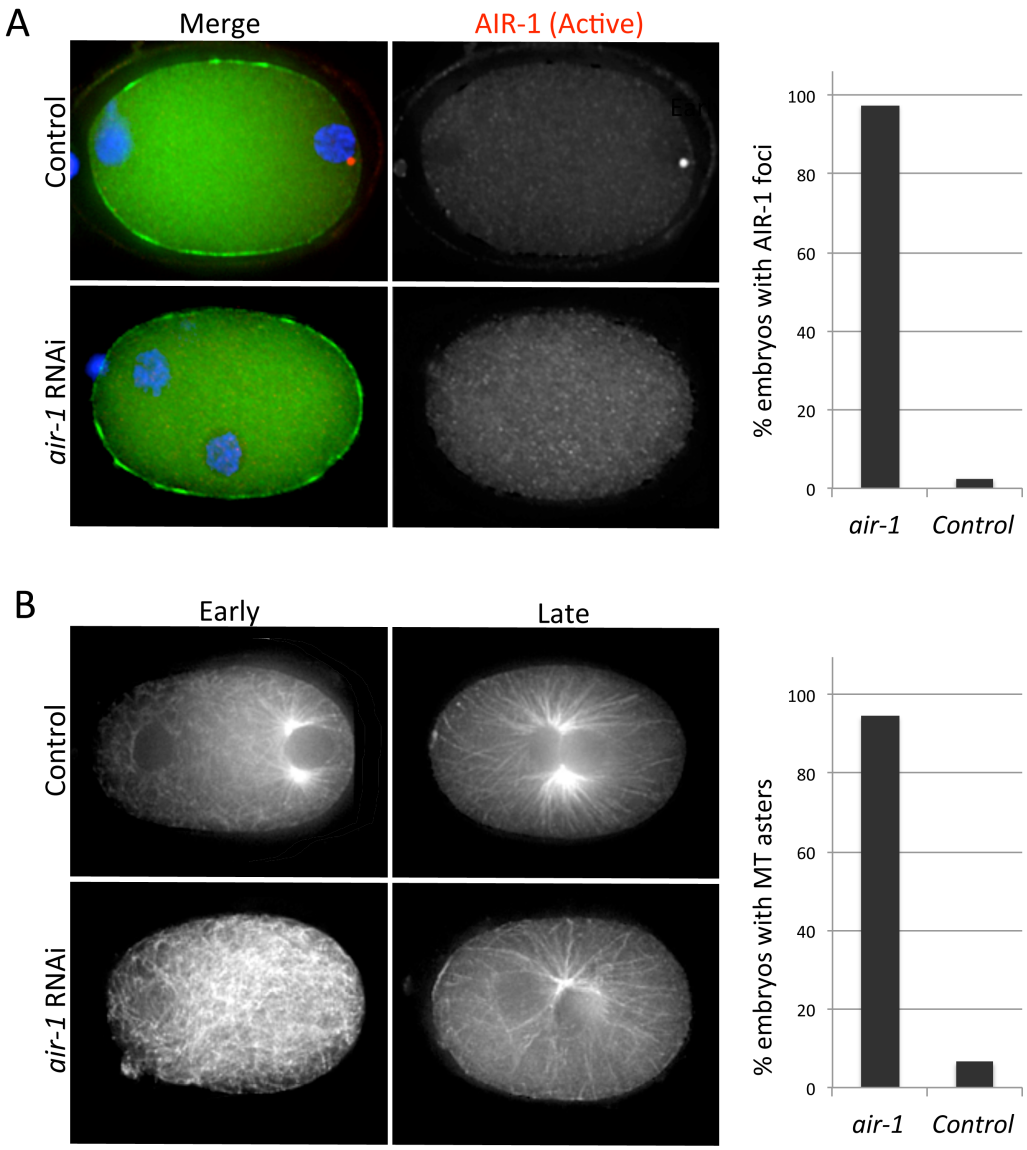


Figure 28. Variable *air-1* phenotypes are not likely related to partially effective RNAi. A. *air-1* RNAi induces loss of AIR-1 PO4 staining (active) in NMY-2::GFP, which in this embryo is completely mislocalized. Similar effects were observed in the PAR-6::GFP strain. The quantification of loss of AIR-1 staining following *air-1* RNAi is shown at the right. B. *air-1* RNAi also leads to loss of microtubule asters. In early embryos, when asters are normally separated and localized to each side of the paternal pronucleus, *air-1* RNAi treated embryo lack asters, and instead, show short microtubules localized throughout the embryo. This effect is quantified in the graph at the left. In late stage *air-1* treated embryos (pronuclear meeting), microtubule asters are observed, although these are highly disorganized. Centrosomes also fail to separate, leading to mitotic defects.

in cell cycle, pronuclear migration, and chromosome condensation that may result from *air-1* RNAi (Portier et al., 2007; Hachet et al., 2007).

We were intrigued to note the consistent variability associated with *air-1* RNAi, and we hypothesized that this might be suggestive of an additional, partially redundant polarizing pathway. An attractive candidate to function such a pathway is the posterior localized RING-finger protein PAR-2. First, we characterized the *par-2* RNAi phenotype during the early stages of embryogenesis. Previous studies (Cuenca et al., 2003; Kemphues and Strome labs) found that PAR-2 functions in the maintenance phase of polarity, rather than the initiation phase. By performing RNAi using highly stringent conditions (injection at >2 mg/ml RNAi, and 25^{o}C incubation of P_0 until ~20 % sterility prior to dissection of embryos), we found that loss of PAR-2 function induced ~20% overextension of PAR-6::GFP, and ~50% overextension of NMY-2::GFP. These effects are shown in Figure 29. A previous study (Tsai & Ahringer, 2007) found that the relative levels of anterior and posterior PAR proteins can affect the polarity phenotypes, and this may contribute to these affects. However, we also observed a proportion of embryos with defects in microtubule aster formation, including weak nucleation of microtubules, misaligned or delayed localization of the paternal pronucleus at the posterior cortex relative to chromosome condensation and pronuclear size. A previous study also showed that PAR-2 localizes transiently to the anterior cortex during meiosis (Boyd et al., 1996); this may contribute to the reversed or bilateral phenotypes.

We hypothesized that AIR-1/centrosomal initiation of polarity functions in a common pathway with Rho-1/RhoA GTPase, CYK-4 (GAP), and actomyosin dynamics. To test the hypothesis that PAR-2 functions in polarity initiation, we performed double

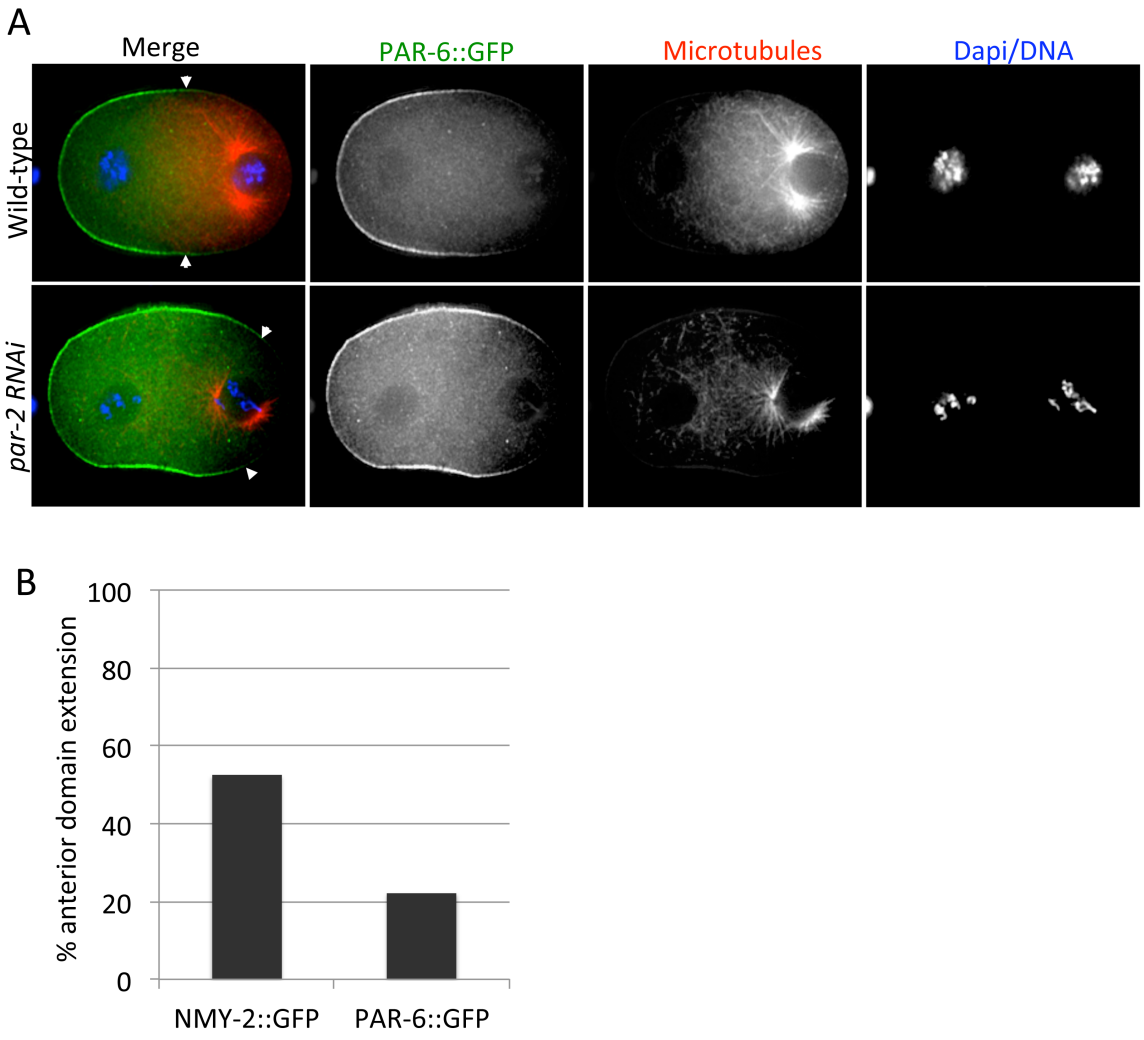


Figure 29. PAR-2 plays a subtle role during polarity initiation. A. *par-2* RNAi induces extension of the PAR-6::GFP anterior domain, as well as reduced astral microtubules and delay in pronuclear migration. B. Quantitation of *par-2* RNAi polarity defects in lines expressing GFP marked anterior factors.

RNAi epistasis experiments by injecting *par-2* and *air-1* or *rho-1* dsRNA in equal concentrations. Control experiments were performed by injecting each RNAi in combination with an off target RNAi (*pha-4*), to ensure equivalent targeting and interaction with RNAi machinery. Embryos were dissected from NMY-2::GFP or PAR-6::GFP P_0 worms injected with 1 mg/ml each RNAi, and fixed, stained, and imaged. In

addition to scoring polarity phenotypes, comarkers (i.e., microtubules, PAR-2) and Dapi/DNA were used to verify effective depletion of targeted genes. For *air-1* RNAi, only embryos lacking microtubule asters were scored. PAR-2 staining was used to confirm the absence of protein in embryos scored in *par-2* RNAi treatment. Following treatment with *rho-1* RNAi, actomyosin foci are lost (in NMY-2::GFP), and partial failure in meiosis II leads to increased DNA in the oocyte pronucleus (anterior). This phenotype, and the quantitation of the additive/synergistic effects are shown in Figure 30. Embryos treated with *air-1* and *rho-1* single RNAi show similar percentages of complete mislocalization of anterior polarity markers, suggesting these factors may function in a common pathway. *air-1*+*rho-1* double RNAi treated embryos are similar to the single-RNAi treated samples (20-30% complete mislocalization). Embryos treated with *par-2* RNAi show a percentage of partial mislocalization, but no complete mislocalization. However, combining *par-2* RNAi with either *air-1* or *rho-1* induces a dramatic increase in the percentage of complete mislocalization of anterior markers. This synergistic effect demonstrates that *par-2* functions in a pathway parallel to the *air-1/rho-1* pathway of polarity specification. Our double RNAi studies in NMY-2::GFP also demonstrate that *air-1* is epistatic to *rho-1* RNAi with respect to contractile foci formation (data not shown), lending additional support for function in a common pathway. We also observed while *rho-1* RNAi resulted in the absence of NMY-2::GFP contractile foci as previously reported, the addition of *par-2* RNAi led to the surprising phenotype of increased concentration of NMY-2 at the cortex. This finding suggests that PAR-2 may feed in to the polarity pathway at the level of actomyosin. A proposed model for these interactions is shown in Figure 31.

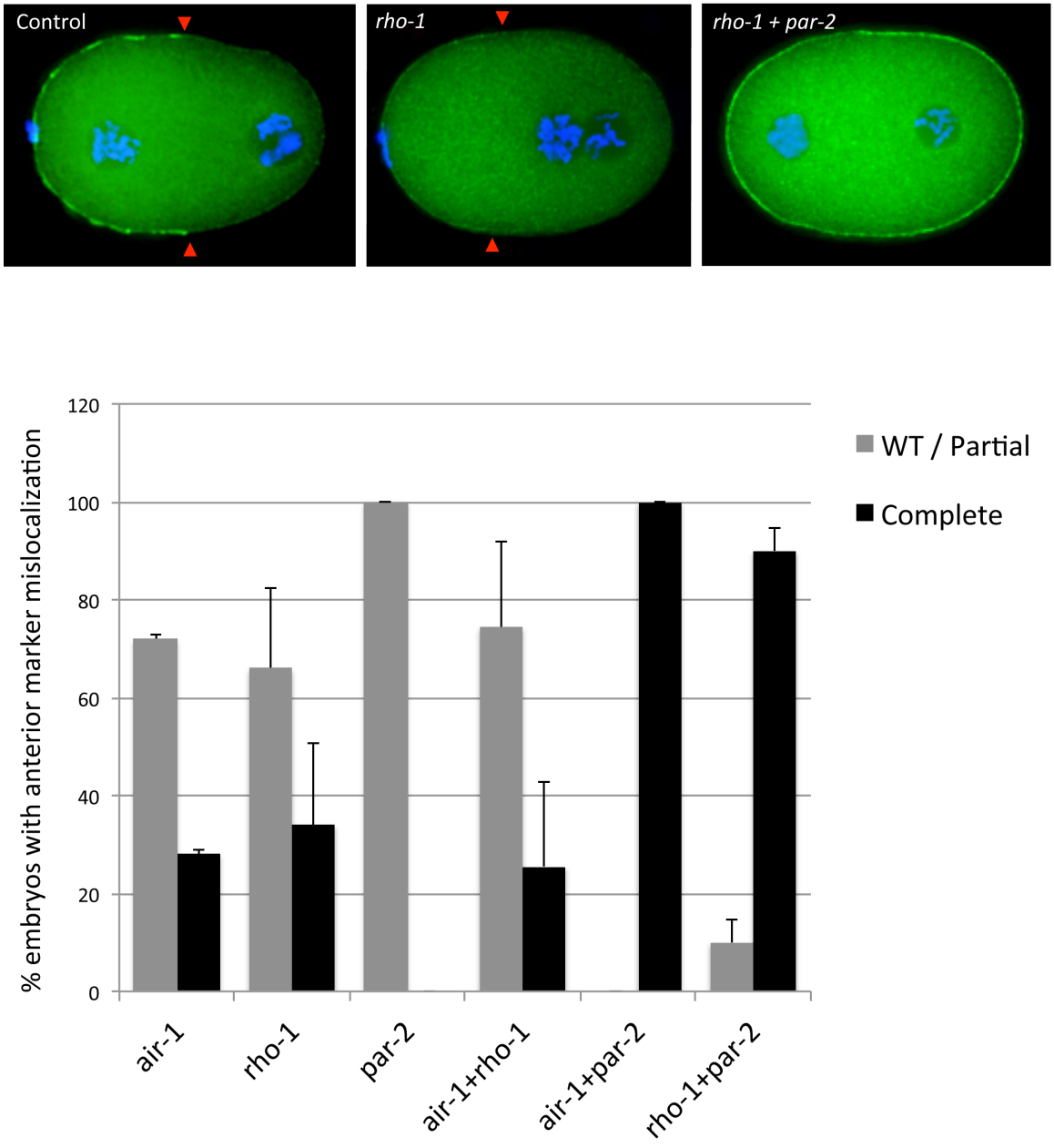


Figure 30. Effects of *rho-1* and *rho-1+par-2* RNAi in NMY-2::GFP embryos. Top: Additive effects of *rho-1+par-2* suggests these factors function in separate pathways. Bottom: Quantitation of *air-1, rho-1, par-2* and combinatorial RNAi demonstrates synergistic effects of *par-2* + *air-1*/*rho-1*.

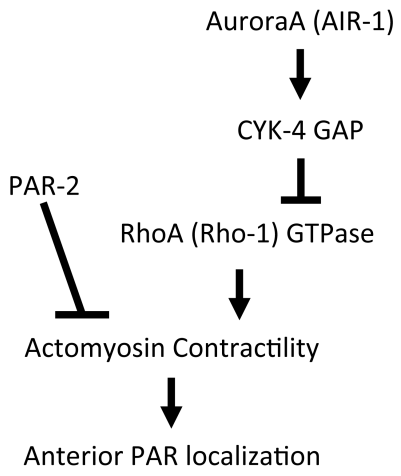
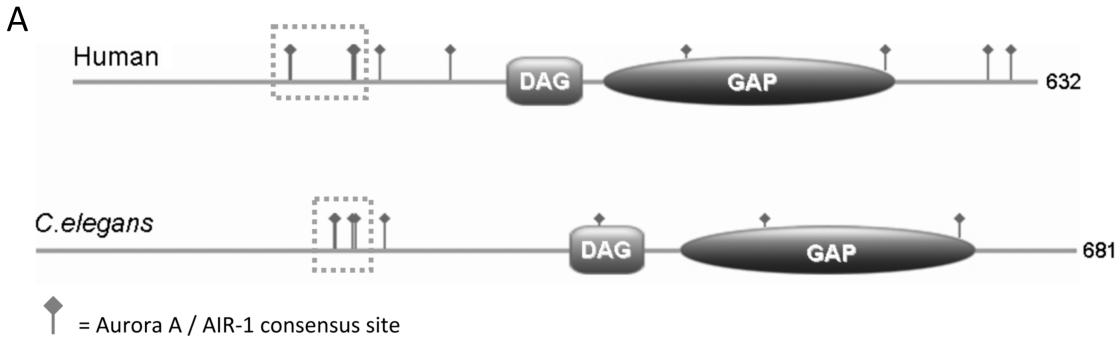


Figure 31. A model of A/P polarity initiation. AIR-1 acts as a centrosomal effector of polarity, acting upstream of CYK-4(GAP), RHO-1/RhoA GTPase and actomyosin dynamics.

Our findings are consistent with a recently published study demonstrating embryos lacking actomyosin activity (by depletion of the regulatory myosin light chain *mlc-4* or the Rho-GEF *ect-2*) are polarized by PAR-2 (Zonies et al., 2010). This study also addressed the antagonism of NMY-2 at the cortex by PAR-2, and showed that PAR-2 indirectly inhibits NMY-2 cortical localization by antagonism of PAR-3 (Zonies et al., 2010). A subsequent study went on to demonstrate an interaction between PAR-2 and the centrosome/astral microtubules (Motegi et al., 2011), including localization of PAR-2 at the MTOC core, and loss of PAR-2 at the cortex when microtubules are absent. PAR-2 was shown to bind microtubules in vitro (Motegi et al., 2011); this activity may account for the destabilization of microtubules and/or failure to properly nucleate astral microtubules. Motegi and colleagues went on to show that microtubule binding protects PAR-2 from aPKC phosphorylation, and that this activity is essential to PAR-2 localization to the cortex and polarity initiation.

We next wanted to address the interaction of AIR-1 and CYK-4, as our hypothesis suggests that AIR-1 kinase activates the GAP activity of CYK-4 toward RHO-1 GTPase. As shown in Figure 32, CYK-4 contains multiple domains that are conserved with mgcRacGAP (the human ortholog of CYK-4). In addition, both proteins contain a number of potential AuroraA/AIR-1 phosphorylation sites, including a cluster of sites in the N-terminal region. The alignment of this region is shown in (B), and the underlined amino acids were found to be required for mcgRacGAP activation by Aurora B kinase



B

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                                197/198          209/211          224/226
C.re   GDSFEEVYTLRNGREVRRSSAAGNPGG-AKRRSASAHAASIAANANSKRSRSRVMAATID 236
C.br   GDSFDECYALRSGREVRRSSAAGNMAGTSKRRSASAHATTAAANS--KRSRSRVMAATID 238
C.bn   GDSFEEVITLRNGREVRRSSAAGNPGG-AKRRSASAHAASTAAAN-SKRSRSRVMAATID 234
C.el   GDSFEEVIHLRNGREVRRSSAAGNAVG-GKRRSASAHAITAAANS--KRSRSRVMTATID 234
C.ja   GDSFDEVITLRNGREVRRSSAAGNHMGGPKRRSASAHAG-AAVNA--KRSRSRAMAATID 234
human  DESLDWDSSLVKTFKLKKR---------EKRRSTSRQFVDGPPGP---VKKTRSIGSAVD 209
mouse  DESLDWDSSLVKNFKMKKR---------EKRRSNSRQFIDGPPGP---VKKTCSIGSTVD 210
       .:*::    * .  ::::           **** * :    .       .::  : :::*
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Figure 32. CYK-4 orthologs in humans and other species. Top panel (A) schematizes conservation of domains in human and *C.elegans*. GAP (GTPase activating) and DAG (diacylglycerol interacting) and AuroraA consensus sites ([KR]XX[ST][ILVGA]) are shown. The boxed regions are shown in B. Lower panel (B), shows alignments of several *Caenorhabditis* species, as well as human and mouse. The AuroraA sequence required for activation during cytokinesis is underlined in red. Additional conserved sites are highlighted in yellow, and the likely equivalents of these in mammalian species are boxed. The S224/226 couplet does not fit the canonical Aurora site defined in mammalian cells, and were not analyzed in our study.

during cytokinesis (Ban et al., 2004). We postulated that these residues (S209/211), and the upstream residues S197/198, which represent conserved Aurora phosphorylation sites, might be targeted by AIR-1 activity and function in A/P polarity initiation. The downstream serine residues (S224/226) are less strong Aurora sites, and were not addressed in our study.

Our collaborators in the Ferrari lab at the University of Zurich (SW) performed in vitro kinase assays using recombinant human Aurora A and GST tagged *C.elegans* CYK-4. These assays demonstrated that Aurora A is capable of phosphorylating CYK-4 (Figure 33). Moreover, deletion of the control region (CR) containing S197, S198, S209 and S211 led to reduced phosphorylation in these assays, supporting our hypothesis. However, as shown in Figure 33, some residual phosphorylation remains, suggesting that

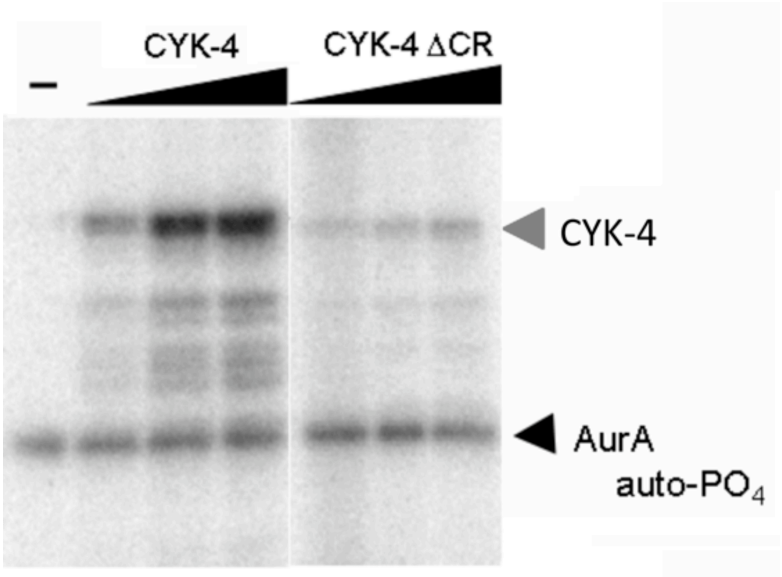

Figure 33. Kinase assay with recombinant proteins suggests that human AurA/AIR-1 phosphorylates CYK-4 at conserved phosphorylation sites (CR, 197/198, 209/211) *in vitro*. (Courtesy of the Ferrari lab).

additional sites may also be phosphorylated *in vitro*, but whether the CR sites or others are phosphorylated in vivo could not be addressed, as attempts to generate and purify a phosphospecific CYK-4 antibody were unsuccessful.

We next addressed whether these conserved Aurora phosphorylation sites (S197/198, S209/211) are essential for CYK-4 function by a transgenic method. Using the *C.briggsae* transgenic system, CYK-4 constructs introduced in single copy by the mosSCI system (as described in Appendix I). We also generated lines containing point mutations to generate phosphomimetic (S→ E and S → D) and phosphodefective (S → A) CYK-4 mutant lines. The serines 197/198 and serines 209/211were mutated in pairs, as in addition, all 4 sites were mutated to alanine (phosphodefective). The different mutant strains are summarized in Table 6. As shown, most strains, including both the wildtype and mutated CYK-4 lines express in both the germ line and embryos (data not shown). We then assessed whether these different mutant constructs were able to rescue the *cyk-4* (*ok1034*) background.

As expected (and discussed in Appendix A), the wildtype *C.briggsae* CYK-4 transgenic was able to fully rescue a *cyk-4* null background. We also predicted that the CYK-4 GAP defective mutations would be unable to rescue the cyk-4 *null*, as the GAP activity is thought to be required for both the polarity and cytokinesis functions of CYK-4. Both GAP domain point mutations (R459A and R459K) result in constructs that express and localize similar to wildtype when the *C.elegans cyk-4* is present (data not shown). However, these constructs do not rescue when crossed into the *cyk-4 (ok1034)* null strain, resulting in sterile adults the homozygous F2 generation, and develop to adulthood based on the maternal contribution from the heterozygous F1. In contrast, strains containing

Table 6. Summary of CYK-4 transgenic constructs and rescue of *cyk-4* loss of function.

Transgenic Construct	Expressing Lines	Rescue *cyk-4$^{C.el}\Delta$*
Wild-type (Control)	3 / 3	Yes
R459A (GAP defective)	1 / 1	No
R459K (GAP defective)	2 / 4	No
S 197/198 A	1 / 1	Yes
S 197/198 D	2 / 3	Yes
S 197/198 E	2 / 2	Yes
S 209/211 A	3 / 4	Yes
S 209/211 D	1 / 1	Yes
S 209/211 E	3 / 5	Yes
S 197/198/209/211 A	2 / 2	Yes

single copies of *C.briggsae cyk-4* which have been mutated to convert S197/198 and/or S209/211 to alanine (phosphodefective) or glutamic/aspartic acid (E/D, phosphomimetic) were able to fully rescue, resulting in Mendelian inheritance patterns by genotyping PCR. These homozygous mutant populations appear fully viable and fertile. However, we did not test GAP activity or of these mutants *in vitro,* nor did we test individual recombinant mutant proteins in Aurora kinase assays. As CYK-4 phosphospecific antibodies could not be generated, we were also unable to test whether these point mutations changed the phosphorylation status. As we were unable to confirm the affects of point mutations with respect to AuroraA/AIR-1, and all mutants (with the exception of GAP defective mutants, which serve as proof of principle) were fully viable, we did not further examine polarity defects in these mutants. However, based on our findings and those of the Seydoux lab, the ability of PAR-2 to act in a separate polarizing pathway may account for the observed

nonlethal phenotypes of these mutants. The requirement for CYK-4 GAP activity during cytokinesis might account for the failure of these mutants to be similarly compensated.

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